AD_____

AWARD NUMBER DAMD17-97-1-7024

TITLE: Effects of Environmental Estrogen on Apoptosis in Normal and Cancerous Breast Epithelial Cells

PRINCIPAL INVESTIGATOR: Matthew Burow

CONTRACTING ORGANIZATION: Tulane University Medical Center
New Orleans, Louisiana 70112

REPORT DATE: May 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1999	Annual (1 May 98 - 30 Apr 99)

4. TITLE AND SUBTITLE

Effects of Environmental Estrogen on Apoptosis in Normal and Cancerous Breast Epithelial Cells

5. FUNDING NUMBERS

DAMD17-97-1-7024

6. AUTHOR(S)

Burow, Matthew

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tulane University Medical Center
New Orleans, Louisiana 70112

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

MCF-7 cells were used to examine the effects of estrogens and environmental estrogens on TNF-induced apoptosis. 17-β-estradiol, o,p'DDT and alachlor were shown to suppress apoptosis in MCF-7 cells and increase expression of Bcl-2. These same compounds were unable to suppress TNF-induced apoptosis of MDA-MB-231 cells suggesting an ER-dependent mechanism of action. To investigate Bcl-2 in TNF resistance by environmental estrogens a Bcl-2 expression vector was transfected into MCF-7 cells and stable clones were established. These MCF-7N/SFFV-BCL-2 clones express higher levels of BCl-2 as compared to vector transfected MCF-7N/Neo cells. The overexpressing MCF-7N/SFFV-BCL-2 cells exhibited a greater resistance to TNF-induced apoptosis as compared to MCF-7N/Neo cells confirming the involvement of Bcl-2 in TNF resistance in these cells. These results have demonstrated the anti-apoptotic potential of selected environmental estrogens in MCF-7 cells. Phytoestrogens have been shown to possess both estrogenic and antiestrogenic activities in a number of systems and certain anti-estrogenic phytochemicals induce apoptosis in MCF-7 cells which may correlate with the protective effects of certain phytochemicals against the development of breast cancer. These results suggest that the ability of environmental estrogens to regulate apoptosis in hormone dependent cells is dependent upon the estrogenic or antiestrogenic nature of the compounds.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Apoptosis, Environmental estrogens, Bcl-2	45
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature May-30-1997 Date

TABLE OF CONTENTS

1. FRONT COVER		**1**
2. STANDARD FORM (SF) 298		**2**
3. FOREWORD		**3**
4. TABLE OF CONTENTS		**4**
5. INTRODUCTION		**5**
6. BODY		**6-11**
	Fig. 1	8
	Fig. 2	8
	Fig. 3	10
7. APPENDIX		**12-13**
	1)- Key accomplishments	12
	2)- Reportable outcomes	
	3)- Manuscripts and Abstracts	13
	A- Unpublished manuscripts	
	B- Carcinogenesis 99	
	C- Cancer Res. 98	

INTRODUCTION

Evidence is accumulating that steroid hormones regulate apoptosis in hormone-responsive tissues. Both prostate and breast epithelial cells undergo apoptosis upon removal of testosterone and estrogen, respectively. This dependence upon hormone for survival and proliferation extends to neoplasms arising from these tissues. The MCF-7 breast cancer cell line has been shown to form tumors in nude ovariectomized mice only in the presence of estrogen. Upon removal of estrogen the tumor cells begin to undergo apoptosis leading to tumor regression. Additionally, two recent studies have shown that pretreatment of MCF-7 cells grown *in vitro* with estrogen reduces the induction of apoptosis by cytotoxic drug treatment as well as tamoxifen. These studies provide evidence that estrogens play a role in both tumorigenesis, as well as drug resistance, through reduction of apoptosis. In addition to estrogen, a class of compounds referred to as environmental estrogens has recently been suggested to play a role in breast cancer. These compounds represent natural or synthetic chemicals that can mimic the activity or effects of endogenous estrogens. The potential exists that these compounds, acting through the estrogen receptor, can affect the apoptotic pathways of estrogen responsive cells. Several recent papers have shown that organochlorine compounds, including DDT, endosulfan and alachlor, act like estrogen and are capable of binding to the estrogen receptor (ER), causing transcription from estrogen response elements (EREs) in DNA, and causing proliferation of MCF-7 cells *in vitro*. Additionally, flavonoid phytochemicals found in diets rich in soy food are often epidemiologically associated with decreased risk of certain hormonally regulated tumors such as breast and prostate. These flavonoid phytochemicals rich diets may function to suppress carcinogenesis in these tissues through their hormonal effects. In regards to the estrogenic action of these phytochemicals , may function both as agonists and antagonist of ER function. Others and we have previously described the estrogenic and anti-estrogenic action of certain flavonoid phytochemicals. Given the importance of estradiol as a proliferative and more importantly as survival factor in breast epithelial cells and the knowledge that clinically used anti-estrogen including tamoxifen are capable of induction of cell death we examined the role of phytochemicals in apoptosis of MCF-7 breast cancer cells. Based on the role of the organochlorine pesticides and phytochemicals as either estrogenic or antiestrogenic compounds suggests a potential effect on the regulation of apoptosis.

BODY

Work during the second year of this pre-doctoral training grant generated significant research and project accomplishments. The doctoral training aspects of this grant resulted in defense of the dissertation work of the principle investigator in October 1998. Subsequent appointment to a post-doctoral research position in the laboratory of Dr. John McLachlan (November 1998) will allow further investigation and completion of the specific aims of this grant. The second year's research focused on completion of key research objectives. Technical objective one focused on assessing the effects of estrogen and various environmental estrogens on regulation of TNF apoptosis in MCF-7 cells. The research to this point has focused on TNF-induced apoptosis and the ability of specific organochlorine pesticides to suppress apoptosis. During the second year the specific environmental estrogens used was expanded to investigate members of the phytoestrogen family. Although not proposed in the initial grant the attention received regarding their potential health benefits in regards to prevention of breast cancer in population which consume diets rich in these flavonoid phytoestrogens has prompted us to further examine the effects of these chemicals on apoptosis. Initial studies performed in collaboration with the laboratory of Dr. John McLachlan revealed certain flavonoid phytochemicals possessed potent antiestrogenic effects. Based upon our previously identified role of estrogen and organochlorine environmental estrogen effects on apoptosis we wished to determine if anti-estrogenic phytochemicals would possess anti-survival effect and promote apoptosis in ER positive MCF-7 breast carcinoma cells. This additional research changes the focus of the initial technical objective to include a broader range of environmental estrogens to be examined. Additionally, task 4 involving investigation of synergistic effects of environmental estrogens has been dropped from the proposed research based upon the retraction of the 1997 Science paper in which the basis for synergistic estrogenic activity of environmental estrogen was described. Additionally, the inability of selected organochlorines to suppress apoptosis in ER negative MDA-MB-231 cells further suggests the survival effects of these chemicals is dependent on an intact ER signaling pathway. Based on these initial findings, the ability of the organochlorine environmental estrogens to increase expression of the pro-survival proto-oncogene Bcl-2 was examined. The assessment of Bcl-2 expression in MCF-7 cells partially completes the tasks under Technical objective 2. The ability of estrogen and environmental estrogens to affect expression of other members of this family will be further characterized in year 3. Mammalian expression vectors for Bcl-2, Bax, Mcl-1 and Bcl-XL were obtained (Task 8). Based upon the estrogen increased expression of Bcl-2 (Task 5) Stable Bcl-2 expressing MCF-7 cells were established and found to be resistant to TNF-induced apoptosis. Other tasks under Technical objectives 3 will be examined based upon further evaluation of estrogen induced expression of other members of the Bcl-2 family. Given the lack of a protective effect of estrogen in MDA-MB-231 cells the expression of members Bcl-2 family with estrogenic agents and the investigation of their role in apoptosis will delayed until studies with MCF-7 cells are complete. Results for research performed during Year 2 are below.

Results

Here we demonstrate that the organochlorine pesticides *o,p'* DDT and alachlor, like 17-β-estradiol, have the ability to suppress tumor necrosis factor alpha (TNF)-induced apoptosis in estrogen receptor (ER) positive MCF-7 breast carcinoma cells (Appendix- Carcinogenesis Manuscript 1999 (Fig 2-3.)). To determine if the anti-apoptotic effects of these chemicals are exclusive to ER positive breast cancer cells we examined their effects on TNF-induced apoptosis on the ER negative MDA-MB-231 breast cancer cell line. TNF (10 ng/ml) treatment induced a 25 % decrease in MDA-MB-231 cell viability from control cells at 24 hours (Appendix- Carcinogenesis Manuscript 1999 (Fig 4.)) Pretreatment of these cells with E_2 (1nm), *o,p'* DDT (100 nM) or alachlor (1μM) for 24 hours prior to the addition of TNF (10 ng/ml) resulted in a 21. 2%, 26.7%, and 23.3 % loss of viability respectively demonstrating that these compounds do not possess survival effects on ER negative breast cancer cells. The ability of these compounds to suppress apoptosis in MCF-7 cells was correlated with an ER-dependent increase in Bcl-2 expression. Taken together these results demonstrate that estrogenic organochlorine pesticides like *o,p'* DDT and alachlor may partially mimic the primary endogenous estrogen, 17-β-estradiol, and function to suppress apoptosis in ER responsive cells (Appendix- Carcinogenesis Manuscript 1999 (Fig 4.)). Immunoblot analysis revealed an increase in expression of Bcl-2 with all three compounds which was inhibited by ICI 182,780 treatment demonstrated this increase was specific to the ER pathway. This suggests that one mechanism for survival effects of estrogenic compounds on MCF-7 cells may be through their ability to increase expression of the Bcl-2 gene. We therefore wished to determine if increased expression of Bcl-2 in the MCF-7 N variant inhibits TNF-induced apoptosis, and if greater expression of Bcl-2 may account for the apoptotic resistance of MCF-7 M and L cells as well as estrogen treated MCF-7 N cells. Mammalian expression vectors containing either a neomycin resistance gene or the human Bcl-2 gene and neomycin resistance gene were transfected into MCF-7 N cells and stably expressing clones were selected in G418. Immunoblot analysis with Bcl-2 antibodies was used to determine expression of Bcl-2 in each clone (Fig. 1). Of the 5 clones examined MCF-7N/Bcl-2 (clone B3) was used to examine the effects of increased Bcl-2 expression on TNF-induced apoptosis. Using trypan blue dye exclusion, viability was assessed in MCF-7N/Bcl-2 (B3) cells versus MCF-7/Vec cells in response to TNF (10 ng/ml) (Fig 2). Over 3 days of TNF exposure MCF-7/Vec cells displayed a time dependent loss of viability (63, 37 and 23 %) as compared to the MCF-7/Bcl-2 (C3) cells which retained viability (90, 87 and 86 %). These results demonstrate that estrogen-mediated cell survival may in part be explained by their ability to increase expression of cell survival genes like Bcl-2.

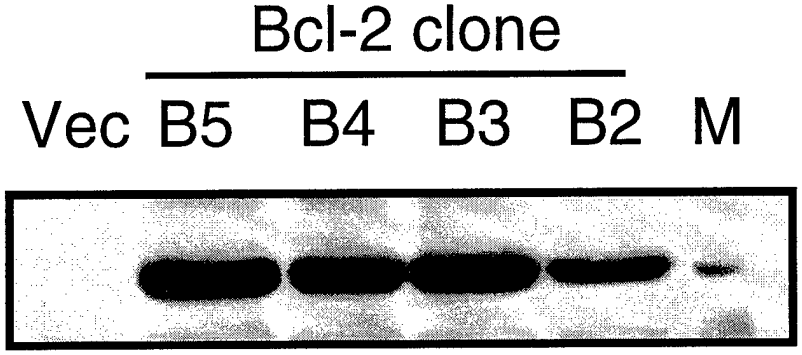


Figure 1. Stable expression of Bcl-2 in MCF-7 N cells. MCF-7 N cells were transfected with SFFV-Bcl-2/neo or SFFV-neo and stable expressing clones were generated by selection in G418 containing 10% DMEM. Individual Bcl-2 expressing clones (MCF-7 N/Bcl-2 (B2-B5)) were selected and confirmed using Western blot analysis with anti-Bcl-2 antibodies, compared to MCF-7 N/vector (Vec) cells or MCF-7 M cells (M).

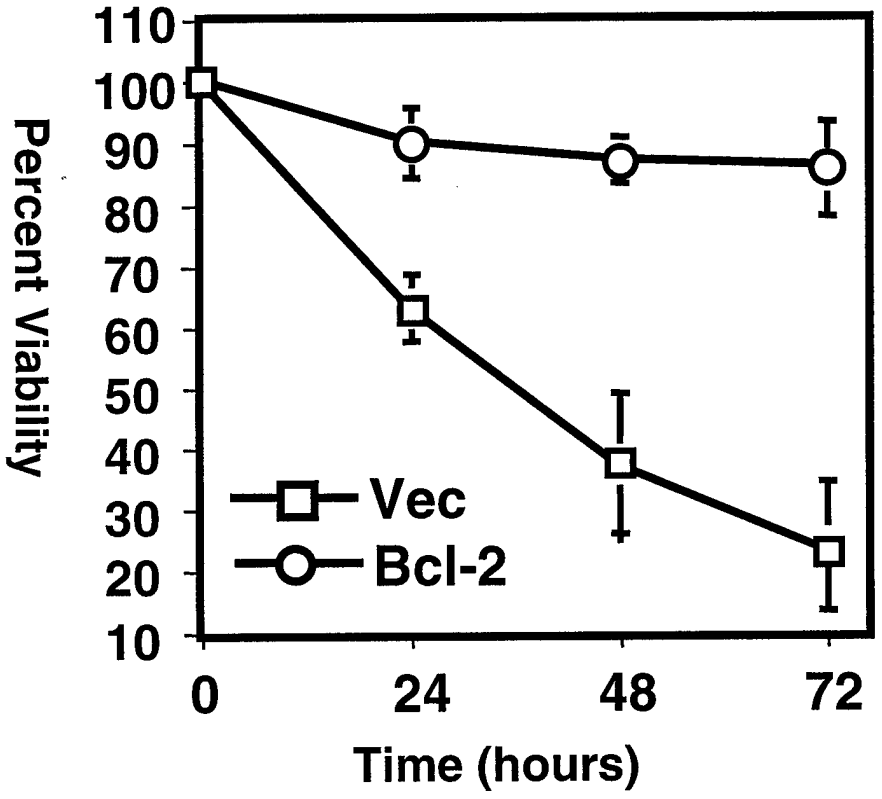


Figure 2. Effects of Bcl-2 expression on TNF-induced apoptosis of MCF-7 N cells. Percent cell death as measured by trypan blue staining at 24, 48 and 72 hours of TNF treatment (10 ng/ml) in MCF-7 N/ Bcl-2 (Clone-3) cells (Bcl-2) or MCF-7 N/Vec cells (Vec). Error bars represent standard error for three experiments

4-OH tamoxifen, the clinically utilized antiestrogen, has previously been shown to induce apoptosis at high concentrations (10uM) whereas at concentrations less than 10uM has been shown to be cytostatic. The ability of a clinically used antiestrogen to induce apoptosis suggested that antagonistic environmental estrogens such as certain phytochemicals might also function to promote apoptosis in sensitive cells. We have previously described the ability of selected phytochemicals including apigenin, luteolin, flavone, kaempferide and chalcone to act as anti-estrogens in breast carcinoma cell and in yeast based estrogen responsive reporter gene analysis (Collins 1997, Collins-Burow – submitted). To determine if these compounds regulate apoptosis, MCF-7 cells were treated with various concentrations of the above phytochemicals or the clinically used 4OH-tamoxifen and viability was assessed using the crystal violet method (data not shown). Consistent with previous results, we observed a dose dependent decrease in cell viability of 4-OH tamoxifen treated MCF-7 cells with concentrations of 10 nM to 10. Both luteolin and chrysin, previously described as estrogen receptor binding dependent antiestrogens (BDA), were assessed for their effects on proliferation/viability. Both luteolin and chrysin lead to an increased proliferation/viability at 1 uM by three days. These two compounds, however, were cytotoxic in MCF-7 cells at concentrations of 10 uM to 50 uM in a time dependent manner. Flavone, apigenin, kaempferide, previously described as estrogen receptor binding independent antiestrogens (BIA), and chalcone were also examined in MCF-7 cells. At a 1 uM concentration all four compounds possessed relatively limited effects on proliferation with flavone and chalcone stimulating proliferation above control at days 3 and 5 and day 3 respectively. Both flavone and apigenin were cytostatic at 10 uM and exhibited cytotoxic effects at 25 and 50 uM concentrations as early as three days. The effects of chalcone and kaempferide on cell death were more rapid in MCF-7 cells. Both compounds induced a time dependent loss of viability at 10 uM. At higher concentrations (25-50 uM) both chalcone and kaempferide exhibited a rapid cell death apparent as early as 24 hours.

To examine if the loss in viability induced by the phytochemicals was due to apoptosis we used DNA fragmentation analysis. MCF-7 cells were treated with phytochemicals at 25 uM concentrations for 72 hours and harvested for DNA fragmentation analysis (Fig 3A). Consistent with the viability data, no DNA fragmentation was observed in vehicle (DMSO), 4-OH-tamoxifen treatment (100nM) (not shown), or coumestrol (25 uM) treated cells at 72 hours (Fig 3A). TNF-α (10 ng/ml), previously shown to induce DNA fragmentation of MCF-7 cells, resulted in significant DNA fragmentation. Consistent with the viability data at 72hrs: kaempferide (32.4+/- 1.4% viable), flavone (54.4+/-10.6%viable), chrysin (30 +/- 1.2% viable), chalcone (12.2+/-1.4% viable), apigenin (23.3+/-0.2% viable), and luteolin (28.2+/-4.8% viable) (not shown) all at 25 uM resulted in DNA fragmentation at 72 hours(Fig 3A). Narigenin, at 25 uM (82.6+/- 4.5% viable) did not induce DNA fragmentation at 72 hours (data not shown). The ability of these compounds to induce apoptotic DNA fragmentation at 50 uM

concentrations at 72 hours was also examined (Fig 3B). Coumestrol treatment (67.4+/-1.0% viable) did not result in DNA fragmentation. DNA fragmentation was observed in MCF-7 cells treated with kaempferide (23.2 +/- 7.9 % viable), flavone (53+/- 0.9% viable), chrysin (23.6+/- 1.9% viable), apigenin (21.2+/-2.1% viable) and chalcone (14.9+/-1.0% viable) at 50 uM for 72 hours. Flavone was the only compound in which treatment resulted in DNA fragmentation without a concurrent loss of greater that 65 % viability at 72 hours at either 25 or 50 uM.

FIG3A

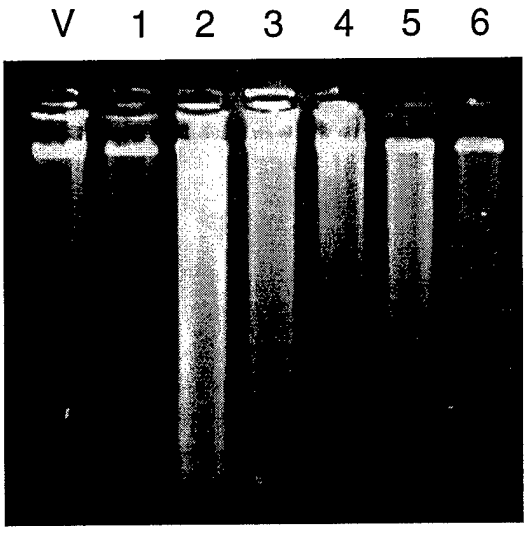


FIG3B

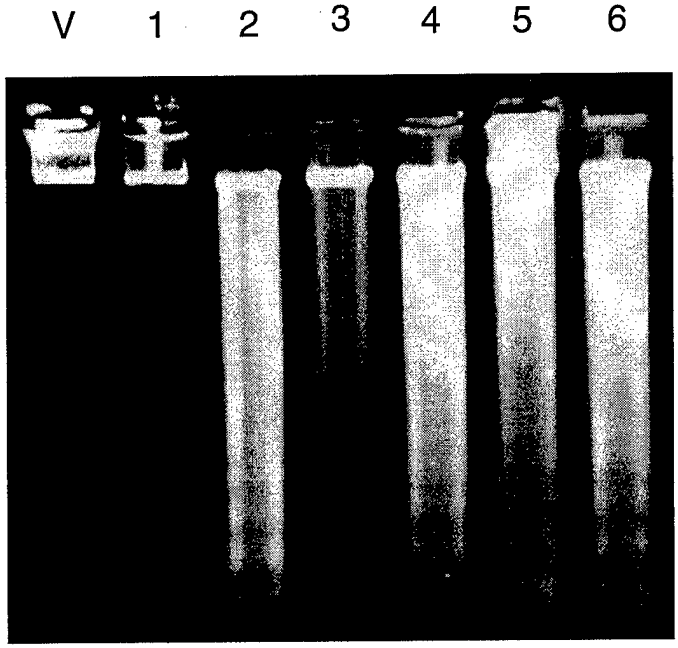


Figure 3. Phytoestrogen-induced apoptosis in MCF-7 breast carcinoma cells. **A**, Cells were treated with either DMSO (V), coumestrol 25 uM (1), 10 ng/ml TNF (2), kaempferide 25 uM (3), chalcone 25 uM (4), chrysin 25 uM (5), or biochanin A 25 uM (6) for 72 hours an harvested for DNA fragmentation analysis. **B**, Cells were treated with either DMSO (V), coumestrol 50 uM (1), kaempferide 25 uM (2), flavone 50 uM (3), chalcone 50 uM (4), chrysin 50 uM (5), or apigenin 50 uM (6) for 72 hours an harvested for DNA fragmentation analysis.

We have demonstrated that certain organochlorine pesticides can suppress apoptosis in ER responsive MCF-7 cells but not in the ER negative MDA-MB-231 cells. The ability of these compounds to suppress apoptosis is correlated with enhanced Bcl-2 expression. Further examination of certain anti-estrogenic plant environmental estrogens revealed opposite activity the estrogenic organochlorine pesticides. Our data here demonstrate that the effects of hormonally active environmental agents on apoptotic signaling are dependent upon their estrogenic anti-estrogenic activity.

Part 7. Appendix to Summary-
Subsection 1) Key Research Accomplishments- Year 2

- Completion of Tasks 1-3 in MCF-7 (ER +) and MDA-MB-231 (ER-) cells.
- Expansion of specific aim 1 to include examination of the flavonoid phytochemical environmental estrogens.
- Examination of Bcl-2 expression and effects on apoptosis in MCF-7 cells. (Tasks 5, 6, 8, 9)
- Completion of dissertation research

Subsection 2) List of reportable outcomes- Year 2

July 98- Presentation: Tulane University - Morris F. and Margaret Shaffer Award for Excellence in Research

Oct. 98- Dissertation Defense: "Molecular Mechanisms of Survival Signaling and Suppression of Apoptosis in Human Breast Carcinoma Cells"

Nov. 98- Publication of a Manuscript: **Burow et al.** Differences in Susceptibility to Tumor Necrosis Factor-α-Induced Apoptosis Among MCF-7 Breast Cancer Cell Variants. *Cancer Research* 1998.

Nov. 98- Post-Doctoral research position (Laboratory of Dr. John McLachlan)

Mar. 99- Acceptance of a manuscript for publication: **Burow et al.** Effects of environmental estrogens on tumor necrosis factor alpha mediated apoptosis in MCF-7 cells. *Carcinogenesis* 1999.

Part 7. Appendix to Summary-
Subsection 3- Cited Manuscripts
-Unpublished Manuscripts

Matthew E. Burow, Yan Tang, Bridgette M. Collins-Burow, Stanislaw Krajewski, John C. Reed, John A. McLachlan, Barbara S. Beckman. Effects of environmental estrogens on tumor necrosis factor alpha mediated apoptosis in MCF-7 cells. Accepted for Publication in Carcinogenesis 1999. (attached below in manuscript submission format)

Bridgette M. Collins-Burow*, **Matthew E. Burow***, Christopher B. Weldon, and John A. McLachlan. Flavonoid phytochemical-induced apoptosis in human breast carcinoma cells via a caspase mediated pathway. Manuscript in preparation. (*Indicates co-authorship)

Bridgette M. Collins-Burow, **Matthew E. Burow**, Bich Duong, John A. McLachlan. The estrogenic and antiestrogenic activities of flavonoid phytochemicals through binding dependent and independent mechanisms. Submitted to Cancer Research June 1999.

Part 7. Appendix to Summary-
Subsection 3- Cited Manuscripts
-Attached Publications/Manuscripts

-Cancer Research Nov. 1998 (Reprint)

-Carcinogenesis 1999 (Manuscript submission format)

Effects of environmental estrogens on tumor necrosis factor alpha mediated apoptosis in MCF-7 cells

Matthew E. Burow[1,2,3,4], Yan Tang[2,4], Bridgette M. Collins-Burow[1,3], Stanislaw Krajewski[6], John C. Reed[6], John A. McLachlan[1,2,3,5], Barbara S. Beckman[1,2,3,4]

Tulane University School of Medicine
Molecular and Cellular Biology Program[1]
Department of Pharmacology[2]
Tulane/Xavier Center for Bioenvironmental Research[3]
Tulane Cancer Center[4]
Department of Environmental Health Sciences[5]

The Burnham Institute[6]
La Jolla, CA 92037-1062

To whom correspondence should be addressed:
Dr. Barbara S. Beckman
Tulane University Medical Center
Department of Pharmacology SL-83
1430 Tulane Ave.
New Orleans, LA 70112
Phone: 504-584-2631
Fax: 504-588-5283
E-mail: bbeckman@tmcpop.tmc.tulane.edu

Running Title: **Environmental estrogens and apoptosis**

*Abbreviations: ER, estrogen receptor; ERE, estrogen response element; TNF, tumor necrosis factor alpha;

Abstract

Environmental estrogens represent a class of compounds which have been shown to mimic the effects or activity of the naturally occurring ovarian hormone 17-β-estradiol. Given the role of 17-β-estradiol in cell survival, we wished to determine if environmental estrogens protect MCF-7 cells from apoptosis. Here we demonstrate that the organochlorine pesticides *o,p'* DDT and alachlor, like 17-β-estradiol, have the ability to suppress tumor necrosis factor alpha (TNF)-induced apoptosis in estrogen receptor (ER) positive MCF-7 breast carcinoma cells. These compounds, however, did not affect TNF-induced apoptosis of the ER negative MDA-MB-231 cell line. The ability of these compounds to suppress apoptosis in MCF-7 cells was correlated with an ER-dependent increase in Bcl-2 expression. Taken together these results demonstrate that estrogenic organochlorine pesticides like *o,p'* DDT and alachlor may partially mimic the primary endogenous estrogen, 17-β-estradiol, and function to suppress apoptosis in ER responsive cells.

Introduction

Apoptosis is a process of cellular suicide by which specific cells undergo a programmed series of biochemical events culminating in the elimination of those cells (1, 2). Apoptosis is a normal physiological process that functions to control cell populations during embryogenesis, immune responses, hormone withdrawal from dependent tissues, and normal tissue homeostasis (1-6). Recent studies have suggested that apoptosis may play a critical role in the generation and progression of cancer and may have potential applications in cancer therapy (1, 4-7).

Accumulating evidence suggests that steroid hormones regulate apoptosis in hormone-responsive tissues. Both prostate and mammary epithelial cells undergo apoptosis upon removal of testosterone and estrogen, respectively (3, 8-11). This dependence upon hormone for survival and proliferation extends to neoplasms arising from these tissues (3, 9, 12, 13). The MCF-7 breast cancer cell line has been shown to form tumors in nude ovariectomized mice only in the presence of estrogen. Upon removal of estrogen the tumor cells begin to undergo apoptosis leading to tumor regression (12). Additionally, recent studies have shown that pretreatment of MCF-7 cells grown *in vitro* with estrogen reduces the induction of apoptosis by cytotoxic drug treatment as well as tamoxifen (14-16). These reports also show that one mechanism by which estrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis regulating proteins whose expression has been shown to suppress MCF-7 cell apoptosis (17). These studies provide evidence that estrogens may play a role in both tumorigenesis, as well as drug resistance, through a suppression of apoptosis.

Environmental estrogens represent a class of compounds both natural or synthetic, which can mimic the function or activity of the endogenous estrogen, 17-β-estradiol. These environmental estrogens may function as endocrine disrupters both in wildlife and humans leading to developmental defects, disease and potentially cancer (18-23). Recently, a number of organochlorine pesticides including DDTs, endosulfan and alachlor, have been shown to mimic estrogen and are capable of binding to the estrogen receptor (ER*), causing transcription from estrogen response elements (ERE) in DNA, and causing proliferation of MCF-7 cells *in vitro* (24-26). The potential exists that these compounds, acting through the estrogen receptor, can affect the apoptotic pathways of estrogen responsive cells. With mounting evidence for the role of estrogen in the regulation of apoptosis, we suggest that these environmental estrogens can act like endogenous estrogen to inhibit TNF-induced apoptosis in ER positive breast cancer cells.

Materials and Methods

Cell Culture

MCF-7 cells (N Variant-Passage 50) were generously provided by Louise Nutter Ph.D. (University of Minnesota)(27). MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD) . MCF-7 and MDA-MB-231 cells were routinely maintained and grown in high glucose containing DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal bovine serum), BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (GibcoBRL, Gaitherburg MD) and porcine insulin $1X10^{-10}$M (Sigma, St. Louis, MO) (10%-DMEM). Cells were maintained in $75cm^2$ tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Prior to experiments both cells lines were placed in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS) supplemented with BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (GibcoBRL, Gaitherburg MD) (5%-CS-DMEM) for 48 hours prior to plating. The cells were plated in 6-well plates at 5 X 10^5 cells/well in the same media and allowed to attach overnight. Following this, cells were treated accordingly. 17-β-estradiol was purchased from Amersham Corporation (Arlington Heights, IL), *o,p'* DDT [1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] was purchased from Sigma Chemical Co. (St. Louis, MO), alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide] and endosulfan II, [(3alpha,5abeta,6beta,9beta,9abeta)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-Methano-2,4,3-benzodioxathiepin 3-oxide] were purchased from AccuStandard (New Haven, CT).

Luciferase Assays

Estrogen responsive reporter gene analysis was performed as described by Klotz et al. (26). Briefly, MCF-7 cells were placed in 5%-CS-DMEM for 48 hours prior to plating. The cells were plated in 6-well plates at 5 X 10^5 cells/well in the same media and allowed to attach overnight. The next day the cells were transfected for 5 hours in serum/supplement-free DMEM with 2 μg of pERE2-luciferase plasmid which contains two copies of the vitellogenin ERE linked to the luciferase gene and 1 μg of the pCMV-β-galactosidase plasmid using 12 μl of lipofectamine (Gibco-BRL, MD). After 5 hours, the transfection media was removed and replaced with phenol red-free DMEM supplemented with 5% DCC-FBS containing vehicle, 17-β-estradiol or environmental estrogen and incubated for 18 hours at 37 °C.

After the 18 hours the treatment containing media was removed and 250 μl of 1X lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) was added per well and incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15000g for 5 min. The cell extracts were normalized for protein concentration using the Bio-Rad Reagent following the supplied protocol (Bio-Rad Laboratories, Hercules, CA). For β-galactosidase assays, the cell extract was placed in 500 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol), 100 μl of o-nitrophenyl-β-D-galactopyranoside at 4 mg/ml in Z-buffer added to each reaction and the tubes placed at 37 °C. The addition of 400 μl of 1M Na_2CO_3 terminated the reactions. The β-galactosidase activity of each reaction was measured at an absorbance of 420 nM. Luciferase activity for the cell extracts were determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Viability assay

Cell viability was determined using the trypan blue assay as described previously (27). Briefly, MCF-7 or MDA-MB-231 cells were plated at 5.0 $X10^5$cells/ml in 10 cm^2 wells in 5%-CS-DMEM. The cells were allowed to adhere for 24 hours before treatment with vehicle (1% DMSO), 17-β-estradiol (1 nM), *o,p'* DDT (100 nM) or alachlor (1 μM) for 24 hours. Following this, cells were treated with TNF (10ng/ml) (R&D systems, Minneapolis MN) and harvested 24 hours later for viability analysis using the trypan blue exclusion method. Percent viability was expressed as the percentage of viable cells in treated samples as compared to control viability with 500 cells counted per sample.

DNA Fragmentation Analysis

Following treatment, cells were harvested for DNA as described previously (27). Briefly, 1-2 $x10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS(w/v) pH 7.4] to which RNAse A (100 μg/ml) was added. After incubation for two hours at 37°C proteinase K (0.5 mg/ml) was added and the lysates were heated to 56°C for 1 hour. NaCl was then added (final concentration 1M) and lysates were incubated overnight at 4 °C. Lysates were centrifuged at 15,000 X g for 30 minutes, and nucleic acids in the supernatant were precipitated in 2 volumes of ethanol with 3M Na acetate. Isolated DNA was then separated electrophoresis on 1.5% agarose gels for 2 hours and visualized by ethidium bromide staining.

Immunoblot Analysis

MCF-7 cells (5 $X10^6$) were grown for three days and then harvested in sonicating buffer (62.5 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol. 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mg/ml leupeptin, 25 mg/ml aprotinin) and sonicated for 30 seconds. Following centrifugation at 1,000 x g for 20 minutes, 50 μg of protein was resuspended in sample loading buffer (62.5 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01%

bromophenol blue), boiled for 3 minutes and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with PBS-Tween (0.05%) - 5% lowfat dry milk solution at 4°C overnight. The membrane was subsequently incubated with a solution of rabbit antisera (anti-Bcl-2 1:4000) and incubated for 2 hours at room temperature. Blots were washed in PBS-Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) for 30 minutes at room temperature. Following four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. Fluorograms were quantitated using image densitometry for data acquisition and analysis.

Results

Several reports in the literature have addressed the estrogenicity of endocrine disrupting chemicals on breast cancer cells. Klotz et al. showed that some organochlorine pesticides such as DDT metabolites and alachlor are capable of binding to the ER as measured by tritiated 17-β-estradiol displacement (26). This group also showed that these compounds are capable of driving reporter gene transcription from ERE elements. These studies indicate the role of environmental estrogens in signaling through estrogenic pathways and like 17-β-estradiol, possess the ability to induce proliferation of MCF-7 cells (24-26). Based on these data we examined the estrogenic activity of estrogen and the environmental estrogens *o,p'* DDT and alachlor on MCF-7 cells. Using an estrogen-responsive reporter gene assay, 17-β-estradiol (1nM) treatment was shown to result in a 12.5 fold increase in luciferase activity (Fig. 1). *o,p'* DDT (100nM) resulted in an equivalent 12 fold increase in luciferase activity while alachlor (1μM) induced a 9.1 fold increase in luciferase activity. Addition of the pure anti-estrogen ICI 182,780 with either E_2, *o,p'* DDT or alachlor reduced luciferase activity to 4.8, 1.8, 0.79 and 0.81 fold respectively, indicating the effect of these compounds on ERE mediated transcriptional activity was dependent on the estrogen receptor. These results demonstrate that the two environmental estrogens *o,p'* DDT and alachlor, at the doses 100 nM and 1 μM respectively, activate ERE mediated gene expression roughly equivalent to a 1 nM dose of the ovarian estrogen 17-β-estradiol.

Based on the observation that estrogen is a survival factor in MCF-7 cells we examined the ability of 17-β-estradiol and the environmental estrogens *o,p'* DDT and alachlor to inhibit TNF-mediated cell death. We previously demonstrated that TNF strongly induced apoptosis in MCF-7 cells (27). Cell viability assay was used to assess the anti-apoptotic effect of 17-β-estradiol and the environmental estrogens *o,p'* DDT and alachlor. TNF (10 ng/ml) caused a decrease in

viability from 100 % in control to 33 % +/-4.46 at 24 hours. Pretreatment of MCF-7 cells for 24 hours with 1nM 17-β-estradiol resulted in a 31% inhibition of TNF-induced cell death restoring viability to 64 % +/- 3.1 (Fig. 2). Similarly 100nM *o,p'* DDT reduced TNF cell death by 30.5% restoring viability to 63.5% +/- 4.46. Treatment with 1 mM alachlor was able to inhibit cell death by 24 % restoring viability to 57% +/- 1.33. DNA fragmentation analysis was used to demonstrate that the suppression of TNF-induced loss of viability was due to induction of apoptosis (Fig. 3). Consistent with previous results, TNF-induced strong DNA fragmentation at 48 hours (27). Twenty-four hour pre-treatment with 17-β-estradiol (1nM), *o,p'* DDT (100nm) or alachlor (1μM) all suppressed TNF-induced DNA fragmentation to near control levels.

To determine if the anti-apoptotic effects of these chemicals are exclusive to ER positive breast cancer cells we examined their effects on TNF-induced apoptosis on the ER negative MDA-MB-231 breast cancer cell line. TNF (10 ng/ml) treatment induced a 25 % decrease in MDA-MB-231 cell viability from control cells at 24 hours (Fig 4.) Pretreatment of these cells with E_2 (1nm), *o,p'* DDT (100 nM) or alachlor (1μM) for 24 hours prior to the addition of TNF (10 ng/ml) resulted in a 21. 2%, 26.7%, and 23.3 % loss of viability respectively demonstrating that these compounds do not possess survival effects on ER negative breast cancer cells.

Given that Bcl-2 is an estrogen responsive gene we examined the ability of 17-β-estradiol and the environmental estrogens *o,p'* DDT and alachlor to increase expression of this gene. MCF-7 cells were treated for 48 hours with 17-β-estradiol (1 nM), *o,p'* DDT (100 nM) or alachlor (1 μM) with or without pre-exposure to ICI 182,780 (100nM). Western blot analysis revealed an

increase in expression of Bcl-2 with all three compounds which was inhibited by ICI 182,780 treatment indicative of a specific ER-mediated pathway (Fig. 5).

Discussion

Environmental estrogens represent a class of compounds which possess the ability to mimic the activity and effects of endogenous 17-β-estradiol. In addition to their role as endocrine disrupters in wildlife, these compounds may also affect humans resulting in developmental defects, disease and potentially cancer. 17-β-estradiol induces proliferation of the ER positive MCF-7 breast cancer cell line, and also acts as a survival factor in these cells in response to treatment with the anti-estrogen tamoxifen as well as with chemotherapeutic drugs (14-16). The ability of environmental estrogens to mimic 17-β-estradiol and cause proliferative and estrogenic effects has been previously analyzed in MCF-7 breast cancer cells (24-26). Given the previously described estrogenicity of these compounds and the role of 17-β-estradiol in cell survival we examined the effects of two organochlorine pesticides on suppression of apoptosis in human breast cancer cells.

We have previously demonstrated that TNF acts as a potent inducer of apoptosis in sensitive MCF-7 cells (27). Consistent with the previous demonstration of the survival effect of estrogen, we show that 1 nM 17-β-estradiol is capable of partially suppressing TNF-induced apoptosis in MCF-7 cells. The ability of both *o,p'* DDT and alachlor to protect against TNF-induced cells death in MCF-7 cells closely correlates with the relative estrogenic potential of these compounds. With the most estrogenic compounds in the reporter gene assay E2 (at 1 nM) and *o,p'* DDT (at 100 nM) exerted the greatest survival effects against TNF-induced cell death. While alachlor, a less potent ER agonist even at 1 μM, exerted the least effect on suppression of TNF cytotoxicity. However upon analysis of DNA fragmentation induced by TNF we observed a significant reduction in apoptosis by pre-treatment with all three agents suggesting that subtle differences in viability may not directly correlate to qualitative analysis of apoptosis.

Recently Shen et al. demonstrated that the isomer of DDT, *p,p'* DDT, was capable of activating cellular signaling events in ER negative MCF-10A cells, suggesting that some organochlorine pesticides or potentially environmental estrogens may function through other signaling pathways (28). To investigate the possibility that either alachlor or *o,p'* DDT affects apoptosis in an ER independent manner we used the ER negative MDA-MB-231 cell line. The ER negative MDA-MB-231 breast cancer cells were not as sensitive to the cytotoxic effects of TNF as the MCF-7 cells. As expected, in ER negative MDA-MB 231 cells, E_2 exerted no protective effect against TNF-induced cell death. Similar to the effects of E_2, pre-treatment of these cells with either *o,p'* DDT or alachlor did not affect the ability of TNF to induce cell death, suggesting at the concentration and conditions tested here the effects observed on apoptosis are occurring through an ER dependent pathways. However we can not rule out the possibility that these compounds may possess anti-apoptotic effects not observed here which occur through ER independent mechanism.

Recent reports show that one mechanism by which estrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis regulating proteins (14-16). Additionally, Jaattela et al. have demonstrated overexpression of Bcl-2 in MCF-7 cells resulted in resistance to TNF-induced apoptosis (17). The data presented here show that like estrogen the two organochlorine compounds *o,p'* DDT and alachlor both increase Bcl-2 expression in an ER-dependent manner, suggesting the mechanism of organochlorine suppression of apoptosis is in part mediated through increased expression of Bcl-2.

We have provided evidence that the organochlorine compounds *o,p'* DDT which can function as environmental estrogens are capable of suppressing TNF-induced apoptosis in human breast cancer cell lines and this effect is correlated with an increased expression of Bcl-2. We have also demonstrated that the anti-apoptotic effect of these compounds is observed in ER positive MCF-7 cells but not in ER negative MDA-MB-231 cells, suggesting specificity of this effect for the ER-mediated pathway. Environmental estrogenic compounds which mimic estrogen have been shown to induce proliferative responses and induce expression of estrogen responsive genes and promoters. Here we demonstrate that certain environmental estrogens are also capable of mimicking estrogen in their ability to suppress apoptosis.

Acknowledgments

We wish to thank Drs. William Toscano, Louise Nutter and Stephen M. Hill for their provision of the MCF-7 cell variants. This work was supported by USAMRAA grant DAMD17-97-1-7024 (to M.E.B.) the Cancer Association of Greater New Orleans (to M.E.B., B.S.B.) the Tulane Cancer Center (to B.S.B.), The Tulane/Xavier Center for Bioenvironmental Research (to M.E.B., J.A.M.) and USDA ___ (to B.M.C-B.).

References

1. Kerr, J. F., Winterford, C. M., and Harmon, B. V. (1994) Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, **73**, 2013-26.
2. Walker, N. I., Harmon, B. V., Gobe, G. C., and Kerr, J. F. (1988) Patterns of cell death. *Methods Achiev. Exp. Pathol.*, **13**, 18-54.
3. Kiess, W., and Gallaher, B. (1998) Hormonal control of programmed cell death/apoptosis. *Eur. J. Endocrinol*, **138**, 482-91.
4. Raff, M. C. (1992) Social controls on cell survival and cell death. *Nature*, **356**, 397-400.
5. Williams, G. T. (1991) Programmed cell death: apoptosis and oncogenesis. *Cell*, **65**, 1097-8.
6. Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456-62.
7. Wright, S. C., Zhong, J., and Larrick, J. W. (1994) Inhibition of apoptosis as a mechanism of tumor promotion. *FASEB J*, **8**, 654-60.
8. Ferguson, D. J., and Anderson, T. J. (1981) Morphological evaluation of cell turnover in relation to the menstrual cycle in the "resting" human breast. *Br. J. Cancer*, **44**, 177-81.
9. Hahm, H. A., and Davidson, N. E. (1998) Apoptosis in the Mammary Gland and Breast Cancer. *Endocrine-Related Cancer*, **5**, 199-211.
10. Kyprianou, N., and Isaacs, J. T. (1988) Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology*, **122**, 552-62.
11. Strange, R., Li, F., Saurer, S., Burkhardt, A., and Friis, R. R. (1992) Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development*, **115**, 49-58.

12. Kyprianou, N., English, H. F., Davidson, N. E., and Isaacs, J. T. (1991) Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res.*, **51**, 162-6.

13. Kyprianou, N., English, H. F., and Isaacs, J. T. (1990) Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. *Cancer Res.*, **50**, 3748-53.

14. Teixeira, C., Reed, J. C., and Pratt, M. A. (1995) Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res.*, **55**, 3902-7.

15. Wang, T. T., and Phang, J. M. (1995) Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Res.*, **55**, 2487-9.

16. Huang, Y., Ray, S., Reed, J. C., Ibrado, A. M., Tang, C., Nawabi, A., and Bhalla, K. (1997) Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. *Breast Cancer Res. Treat*, **42**, 73-81.

17. Jaattela, M., Benedict, M., Tewari, M., Shayman, J. A., and Dixit, V. M. (1995) Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene*, **10**, 2297-305.

18. Colborn, T., vom Saal, F. S., and Soto, A. M. (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.*, **101**, 378-84.

19. Safe, S. H. (1998) Interactions between hormones and chemicals in breast cancer. *Annu. Rev. Pharmacol. Toxicol.*, **38**, 121-58.

20. Wolff, M. S., and Weston, A. (1997) Breast cancer risk and environmental exposures. *Environ. Health Perspect.*, **105 Suppl 4**, 891-6.

21. Tyler, C. R., Jobling, S., and Sumpter, J. P. (1998) Endocrine disruption in wildlife: a critical review of the evidence. *Crit. Rev. Toxicol,* **28**, 319-61.

22. Miller, W. R., and Sharpe, R. M. (1998) Environmentl Oestrogens and Human reproductive cancers. *Endocrine-Related Cancers,* **5**, 69-96.

23. Barton, H. A., and Andersen, M. E. (1998) Endocrine active compounds: from biology to dose response assessment. *Crit. Rev. Toxicol.,* **28**, 363-423.

24. Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.,* **103 Suppl 7**, 113-22.

25. Soto, A. M., Chung, K. L., and Sonnenschein, C. (1994) The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ. Health Perspect.,* **102**, 380-3.

26. Klotz, D. M., Beckman, B. S., Hill, S. M., McLachlan, J. A., Walters, M. R., and Arnold, S. F. (1996) Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. *Environ. Health Perspect.,* **104**, 1084-9.

27. Burow, M. E., Weldon, C., Tang, Y., Krajewski, S., Reed, J. C., Clejan, S., and Beckman, B. S. (1998) Differential sensitivity to tumor necrosis factor -induced apoptosis among MCF-7 breast cancer cell variants. *Cancer Res.,* **58**, 4940-4946.

28. Shen, K., and Novak, R. F. (1997) DDT stimulates c-erbB2, c-met, and STATS tyrosine phosphorylation, Grb2- Sos association, MAPK phosphorylation, and proliferation of human breast epithelial cells. *Biochem. Biophys. Res. Commun.,* **231**, 17-21.

Figure Legends

Figure 1. Reporter gene activity of environmental estrogens. MCF-7 cells were transfected with 2 μg of ERE-luciferase containing reporter gene constructs. Cells were then treated with vehicle (control), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT), 1 μM alachlor (Ala) or 1 μM endosulfan II (Endo) in the presence or absence of 100 nM ICI 182,780 (+ ICI). Cells were harvested 18 hours later for luciferase assays. Data is expressed as fold relative light units (RLU's) over control from a representative experiment.

Figure 2. Effects of estrogens on TNF-induced cell death in MCF-7 N cells. MCF-7 cells were treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT), 1 μM alachlor (Ala) for 24 hours prior to the addition of TNF (10 ng/ml) (TNF). Cell were harvested 24 hours later and viability was assessed using the trypan blue method. Error bars represent standard deviation of the mean for 5 independent experiments performed in duplicate. Absence of error bars indicates less than 2.5% S.D.

Figure 3. Effects of estrogens on TNF-induced DNA fragmentation in MCF-7 N cells. MCF-7 cells were treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT), 1 μM alachlor (Ala) for 24 hours prior to the addition of TNF (10 ng/ml). Cell were harvested 48 hours later for DNA fragmentation analysis. Molecular weight marker is shown as MW.

Figure 4. Effects of estrogens on TNF-induced cell death in MDA-MB-231 cells. Cells were treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT), 1 μM alachlor

(Ala) for 24 hours prior to the addition of TNF (10 ng/ml) (TNF). Cell were harvested 24 hours later and viability was assessed using the trypan blue method.

Figure 5. Estrogen-induced Bcl-2 expression. MCF-7 cells were grown in 5% CS-DMEM medium for 5 days and subsequently treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p'* DDT (DDT), or 1 μM alachlor (Ala) in the presence or absence of 100 nM ICI 182,780 (+ ICI) as shown above. Cell were harvested 24 hours later for western blot analysis of Bcl-2 expression.

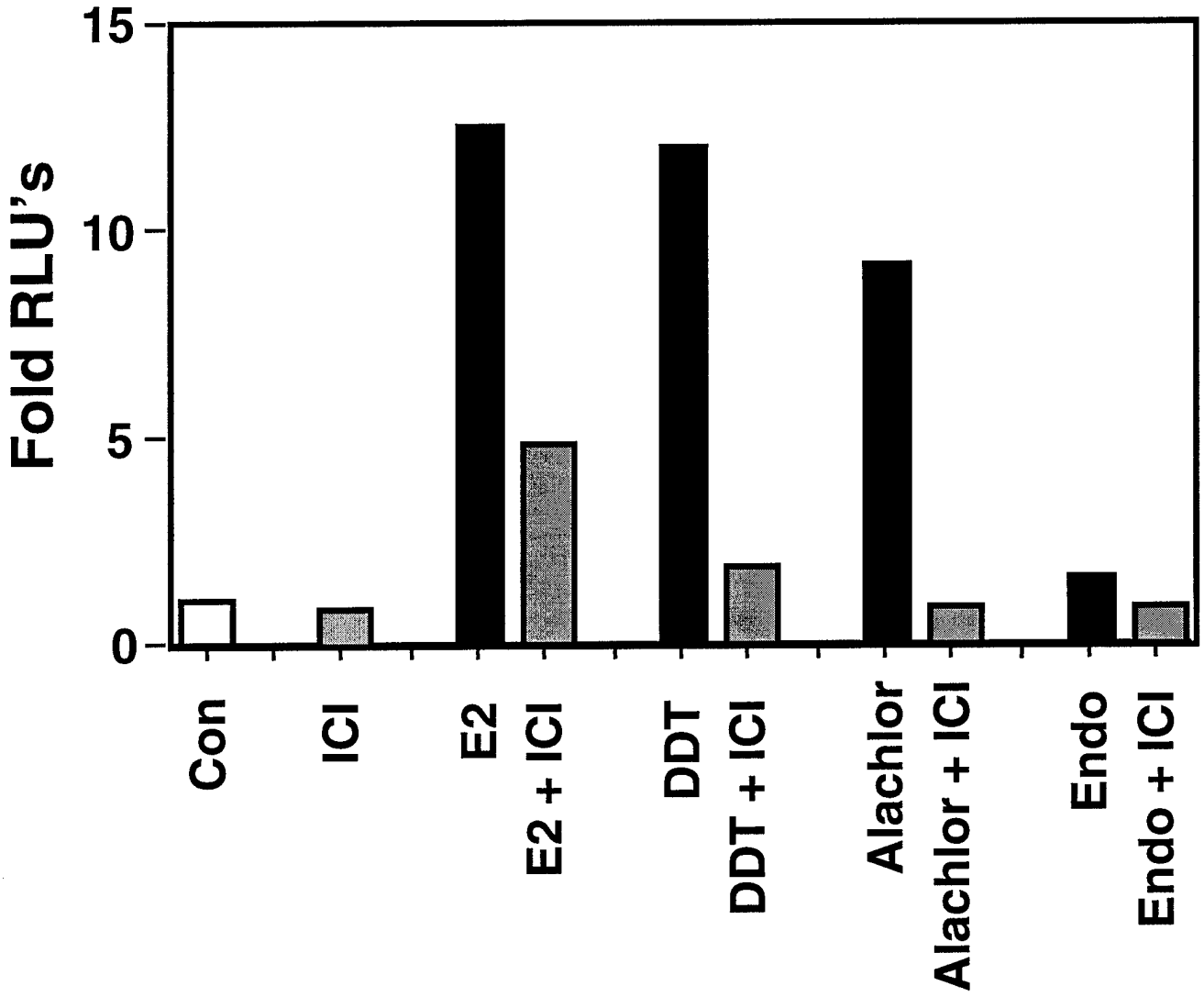
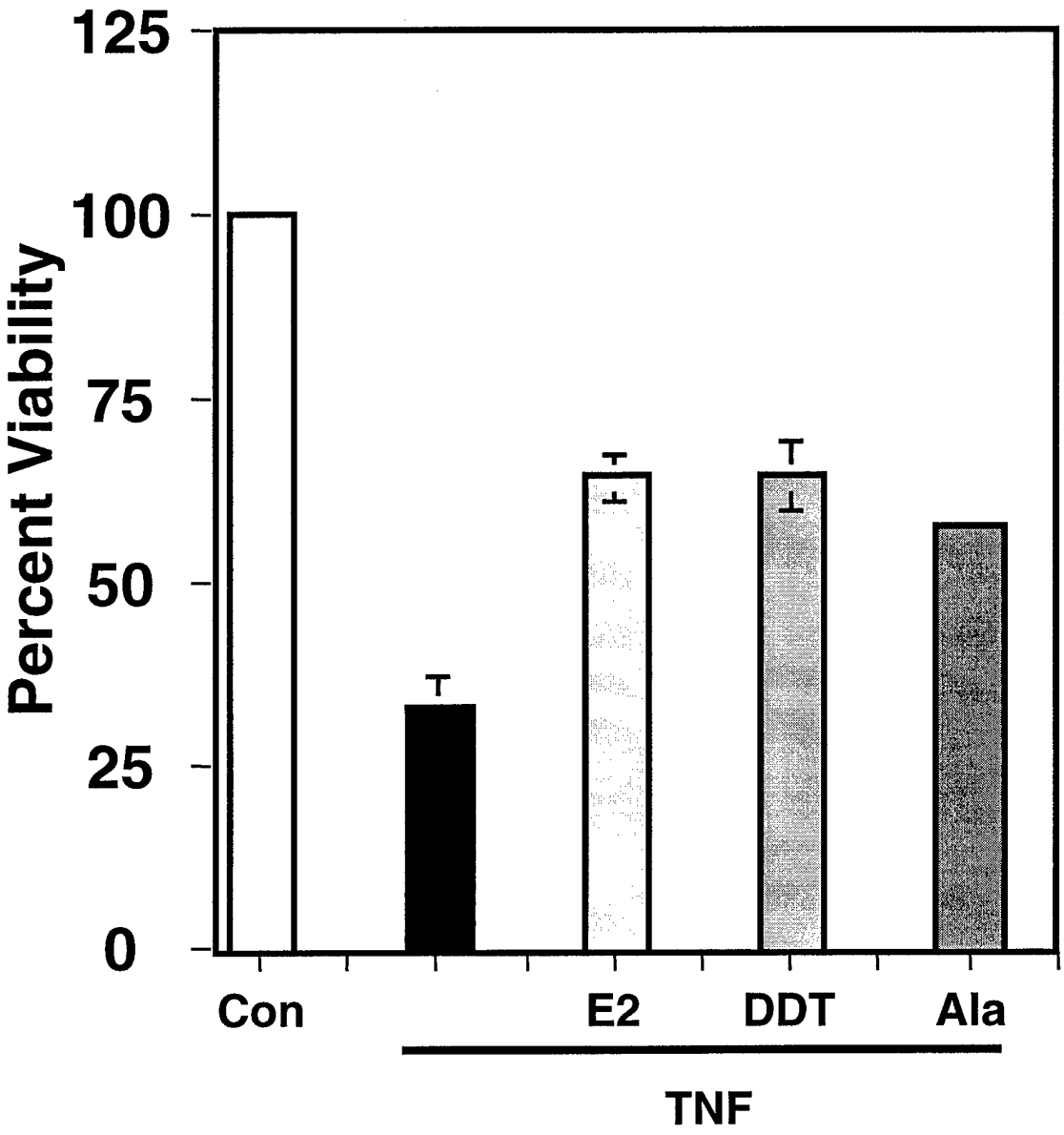
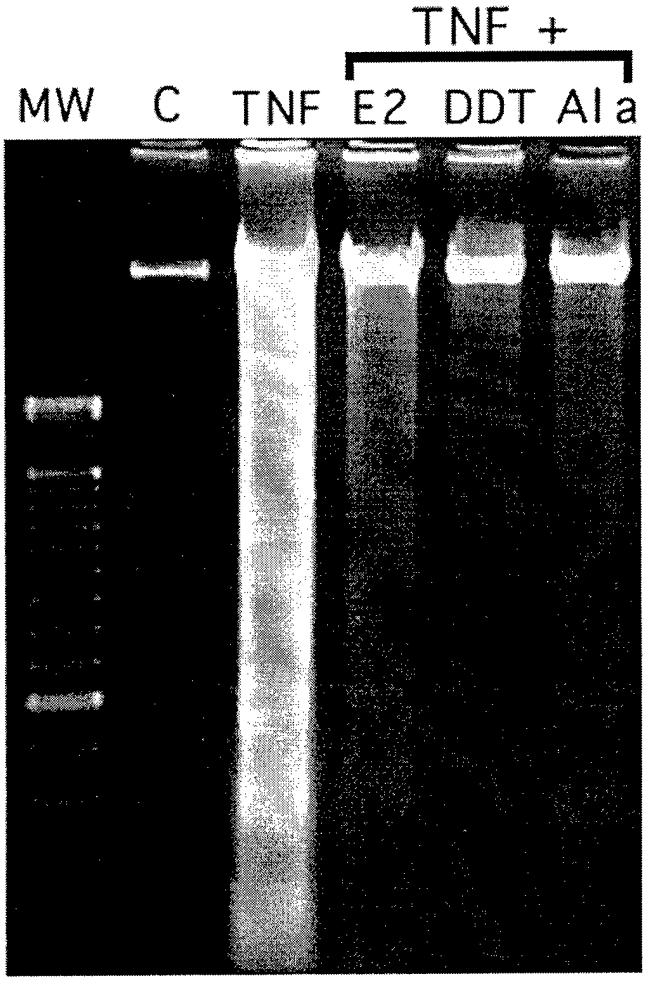
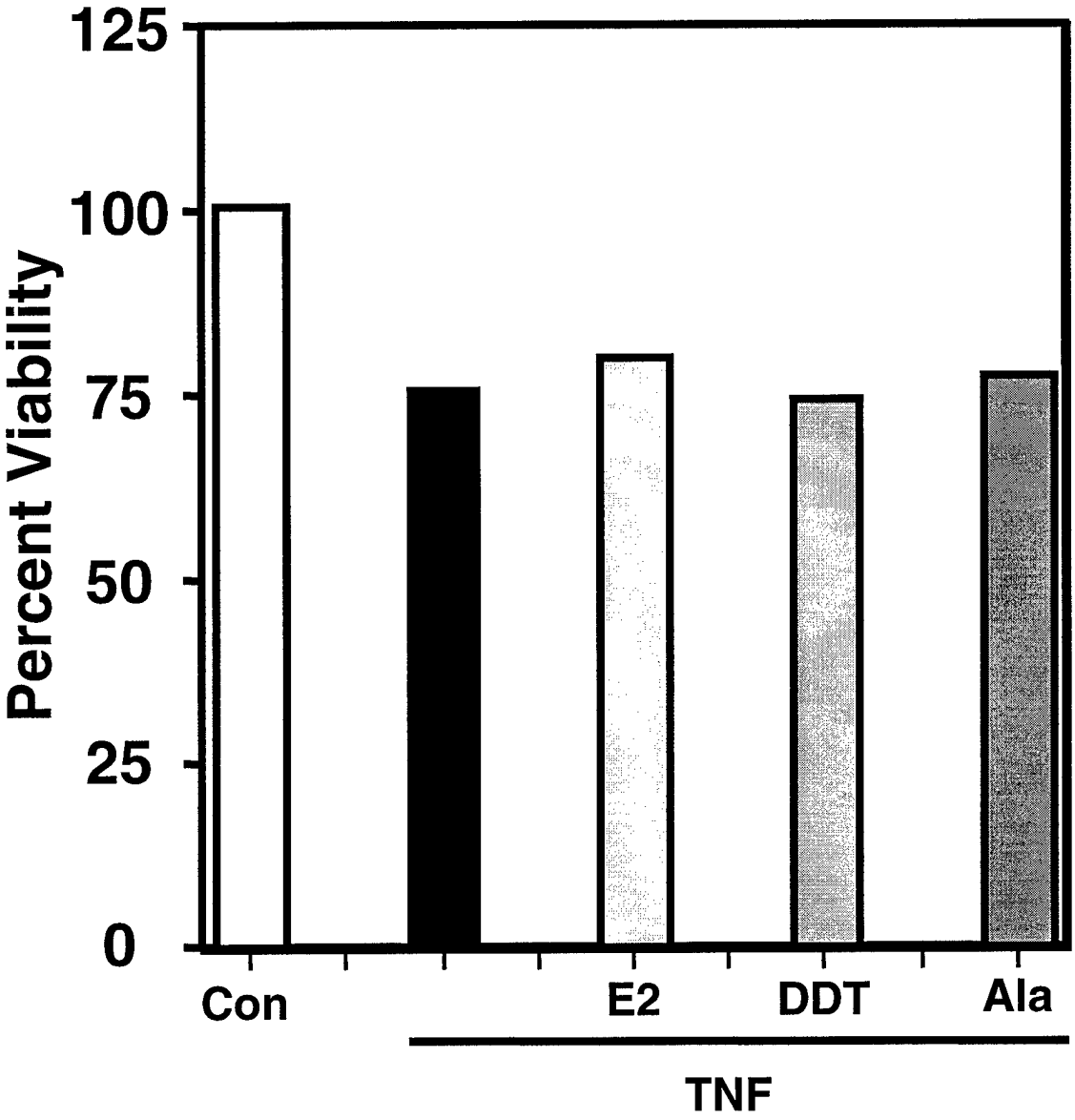
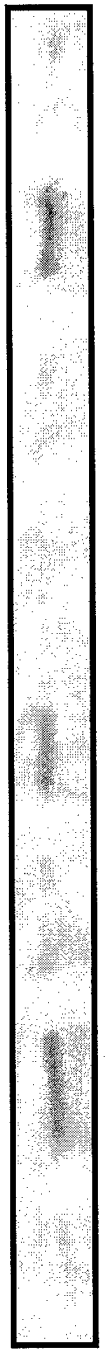


Figure 12. ERE-luciferase activity of environmental estrogens. MCF-7 Cells were transfected with 2 µg of ERE-luciferase containing reporter gene constructs. Following this eld s ewe treated with vehicle (control), 1 nM 17-β-estradiol (E2), 100 nM o,p' DDT (DDT), 1 µM alachlor (Ala) or 1 µM endosulfan II (Endo) in the presence or absence of 100 nM ICI 182,780 (+ ICI). Cells ewe harvested 18 hours after for uciferase assay. Data i sexpressed a sfold relative light units (RLU's) over control rom a representative experiment


125
100
75
50
25
0
Percent Viability
Con
E2
DDT
Ala
TNF


TNF +
MW
C
TNF
E2
DDT
Ala


125
100
75
50
25
0
Percent Viability
Con
E2
DDT
Ala
TNF


C
E2
E2 + ICI
ICI
DDT
DDT + ICI
Ala
Ala + ICI

[CANCER RESEARCH 58, 4940–4946, November 1, 1998]

Differences in Susceptibility to Tumor Necrosis Factor α-induced Apoptosis among MCF-7 Breast Cancer Cell Variants[1]

Matthew E. Burow, Christopher B. Weldon, Yan Tang, Gabriel L. Navar, Stanislaw Krajewski, John C. Reed, Timothy G. Hammond, Sanda Clejan, and Barbara S. Beckman[2]

Molecular and Cellular Biology Program [M. E. B., S. C., B. S. B.], Department of Pharmacology [C. B. W., B. S. B.], Tulane Cancer Center [M. E. B., C. B. W., Y. T., T. G. H., S. C., B. S. B.], Department of Surgery [C. B. W.], Center for Bioenvironmental Research [G. L. N., T. G. H, S. C., B. S. B.], Department of Medicine, Nephrology Section, and Department of Pathology and Laboratory Medicine [S.C.], Tulane University School of Medicine, New Orleans, Louisiana 70112, and The Burnham Institute, La Jolla, California 92037-1062 [S. K., J. C. R.]

ABSTRACT

Widespread use of MCF-7 human breast carcinoma cells as a model system for breast cancer has led to variations in these cells between different laboratories. Although several reports have addressed these differences in terms of proliferation and estrogenic response, variations in sensitivity to apoptosis have not yet been described. Tumor necrosis factor α (TNF-α) has been shown to both induce apoptosis and inhibit proliferation in MCF-7 cells. We observed that TNF-α inhibited proliferation in MCF-7 cell variants from three different laboratories (designated M, L, and N). MCF-7 M cells were resistant to TNF-α-induced apoptosis, whereas MCF-7 L cells were moderately resistant to the effect of TNF-α. A third variant, MCF-7 N, underwent apoptosis when exposed to TNF-α. Analysis of the p55 TNF-α receptor (TNFR) 1 expression revealed the greatest expression in MCF-7 N cells, whereas the MCF-7 L and M cells expressed 89 and 67% of MCF-7 N cell TNFR1 levels, respectively. Ceramide generation occurred in all three variants in response to TNF-α treatment, with MCF-7 N cells expressing the greatest increase. Cleavage of the CPP32/caspase 3 substrate poly(ADP-ribose) was observed in MCF-7 N and L cells as early as 3 and 6 h, respectively, but poly(ADP-ribose) cleavage was not observed in MCF-7 M cells. The delayed protease activation in the L variant may represent the mechanism by which these cells display delayed sensitivity to TNF-α-induced apoptosis. Expression of the Bcl-2, Mcl-1, Bcl-X, Bax, and Bak proteins was analyzed to determine whether the differences in MCF-7 cell sensitivity to apoptosis could be correlated to the differential expression of these proteins. Whereas Bak, Bcl-X, and Mcl-1 levels were identical between variants, the levels of Bcl-2 were 3.5–3.8-fold higher and the levels of Bax were 1.5–1.7-fold lower in the resistant variants (M and L) as compared with those of the sensitive variant (N). Taken together, these results suggest that differences in susceptibility to TNF-α-induced apoptosis among MCF-7 breast cancer cell variants may be explained by differences in TNFR expression, ceramide generation, differential expression of the Bcl-2 family of proteins, and protease activation.

INTRODUCTION

The MCF-7 cell line was established in 1973 from a pleural effusion of a patient with metastatic breast carcinoma who was previously treated with radiation and hormonal therapy (1). Since that time, this cell line has become a model system of ER[3]-positive breast cancers (2). Previous studies suggest that MCF-7 cell line variants possess intrinsic differences in estrogen responsiveness and proliferation rates. Osbourne *et al.* (3) reported that MCF-7 cells obtained from different laboratories varied in proliferation rates, ER and progesterone receptor levels, estrogen and antiestrogen responses, and tumorigenicity. Klotz *et al.* (4) showed that different stocks of MCF-7 cells displayed different levels of variant ER mRNAs, which correlated with their differential response to estrogen stimulation. Different MCF-7 variants tested by Villalobos *et al.* (5) exhibited different responses to 17β-estradiol-induced proliferation and expression of the estrogen-responsive genes *pS2* and *cathepsin D*. These reported variations in MCF-7 cells could potentially lead to contradictory results, depending on the origin of the variant of MCF-7 cells studied.

Apoptosis and apoptotic signaling have recently been examined in MCF-7 cells in response to a number of stimuli including okadaic acid, staurosporine, Fas, retinoic acid, vitamin D analogues, 4-hydroxy-tamoxifen, ceramide analogues, hormone withdrawal, and various chemotherapeutic drugs (6–15). TNF-α is also an effective inhibitor of proliferation and inducer of apoptosis in MCF-7 cells (7, 8, 16, 17). In other studies, MCF-7 cells reportedly responded only weakly to TNF-α (18–21). MCF-7 cells made resistant to TNF-α by continuous passaging in increasing concentrations of TNF-α express decreased levels of TNFR and do not activate SMase or phospholipase A_2 with TNF-α treatment (17). The reported differences in the sensitivity of MCF-7 cells to TNF-α and potentially other apoptotic-inducing agents raised the possibility that variations in MCF-7 cell strains among laboratories may account for these discrepancies.

The effects of TNF-α are mediated through two distinct but related plasma membrane receptors, p55 (TNFR1) and p75 (TNFR2). Both receptors generate distinct biological effects, with the cytotoxic effects of TNF-α being primarily mediated through TNFR1 (22, 23). Although these receptors share limited cytoplasmic homology, they activate some overlapping signaling cascades, such as nuclear factor κB, via the recruitment of specific signaling intermediates to the cytoplasmic domains (22, 23). In the case of TNFR1, TNF-α ligation results in association with TRADD (24), which then recruits TNFR-associated protein 2, receptor-interacting protein (25), and FADD/MORT1 (26), association with the receptor is followed by the association of FLICE/MACH1 with the TNFR1 complex (27, 28). Subsequent to the formation of this protein complex, the activation of several signaling cascades including phospholipase A_2, SMase, nuclear factor κB, stress-activated protein kinases, and apoptotic proteases occurs (22, 23). Activation of SMase, resulting in ceramide formation, represents an early event in the apoptotic signaling cascade (29, 30). MCF-7 cells have been shown to activate SMase in response to TNF-α and undergo apoptosis when exposed to water-soluble ceramide analogues (17). Additionally, studies of acidic SMase knockout mice have shown that ceramide generation may be required for apoptosis by TNF-α and other inducers in some cell types (31).

Received 1/5/98; accepted 9/2/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by USAMRAA Grant DAMD17-97-1-7024 (to M. E. B.); the Cancer Association of Greater New Orleans (M. E. B. and B. S. B.); the Tulane Cancer Center (B. S. B.); NIH Grants R29 DK46117, RO1 DK51392 (to T. G. H.), and 1 T32 CA65436-01A3 (to C. B. W.); and Veteran's Affairs Research Associate Career Development Award LEQSF (1997-98)-ENH-TR-76 (to T. G. H.).

[2] To whom requests for reprints should be addressed, at Department of Pharmacology SL-83, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112. Phone: (504) 584-2631; Fax: (504) 588-5283; E-mail: bbeckman@tmcpop.tmc.tulane.edu.

[3] The abbreviations used are: ER, estrogen receptor; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; PARP, poly(ADP-ribose) polymerase; SMase, sphingomyelinase; TRADD, TNFR1-associated death domain protein; FADD/MORT1, Fas-associated death domain; FLICE, FADD-like ICE; MACH, Mort1-associated CED-3 homologue; ICE, interleukin 1β-converting enzyme; FBS, fetal bovine serum; DAG, diacylgycerol; MTS, 3-(4,5-dimethyl-thiazol-2-yl)-5-3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

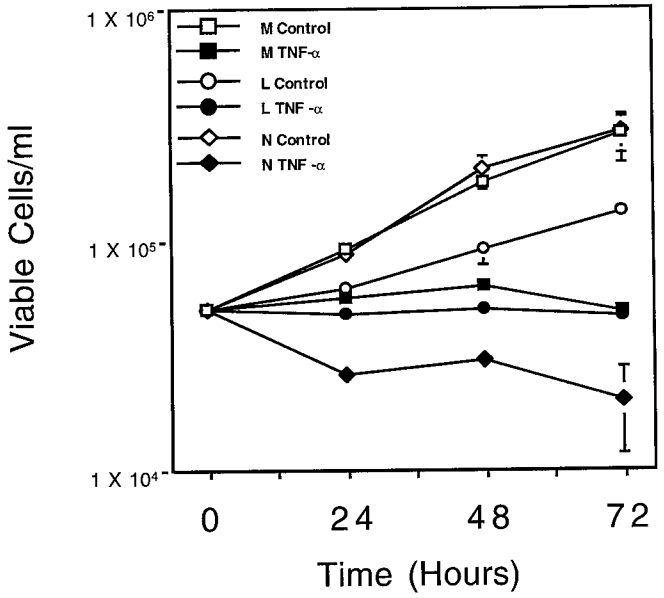


Fig. 1. Effects of TNF-α on the proliferation of MCF-7 cell variants. Each MCF-7 cell variant was plated in DMEM with 10% FBS alone or in the presence of 10 ng/ml TNF-α. Cells were harvested and counted at 24, 48, and 72 h to determine the number of viable cells/milliliter. *Error bars,* SD for duplicate experiments performed in triplicate.

Caspase activation is also thought to represent an early event in TNF-α cell death signaling. The death domain-containing protein FLICE/MACH1/caspase 8 possesses an ICE-like protease domain that becomes activated upon association with the TNFR1-TRADD-FADD complex. The activation of FLICE is thought to result in the subsequent activation of ICE/caspase 1 and CPP32/caspase 3 (32, 33). Therefore, both ceramide generation and protease activation represent potential regulatory points of TNF-α-induced apoptotic signaling.

The Bcl-2 family of proteins comprises a number of related proteins whose expression has been shown to regulate apoptosis (34, 35). This family includes antiapoptotic members (Bcl-2, Mcl-1, and Bcl-X_L) and proapoptotic members (Bax, Bcl-X_S, and Bak) whose individual expression and heterodimerization with each other is believed to regulate the sensitivity of cells to apoptosis. Although the actual biochemical function of these proteins has yet to be completely elucidated, these proteins act upstream of caspase activation through the regulation of cytochrome *c* release from the mitochondria (36, 37).

This study tests directly whether variants in the phenotype of MCF-7 cells may explain the reported differences in susceptibility to apoptosis induced by TNF-α and other agents. The molecular mechanisms for these observations are dissected by examining several events in the signal transduction cascade of TNF-α including TNFR expression and SMase and caspase activation as well as the expression of specific members of the Bcl-2 family of proteins.

MATERIALS AND METHODS

Cell Culture. MCF-7 cell variants M and L were a gift from Stephen M. Hill (Tulane University, New Orleans, LA). The MCF-7 M cell variant (passage 180) originated from the laboratory of the late William McGuire (University of Texas, San Antionio, TX.). The MCF-7 L cell variant (passage 40) originated from the laboratory of Marc Lippman (Georgetown University, Washington, DC). The MCF-7 N cell variant (passage 50) is a subclone of MCF-7 cells from the American Type Culture Collection (Rockville, MD) that was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN). All MCF-7 cells were routinely maintained and grown in DMEM supplemented with 10% FBS, BME (Basal Medium Eagle) amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD), and 1×10^{-10} M porcine insulin (Sigma Chemical Co., St. Louis, MO) under *Mycoplasma*-free conditions.

Proliferation and Viability Assay. MCF-7 cells were plated at 5.0×10^4 cells/ml in 10-cm^2 wells. The cells were allowed to adhere for 18 h before treatment with recombinant human TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN). Cells were then counted at 24, 48, and 72 h posttreatment. The results are represented as the number of viable cells/milliliter as measured by trypan blue exclusion. Apoptosis was expressed as the percentage of trypan blue-stained cells in treated samples compared to control viability. The MTS viability assay (Promega) was performed according to the manufacturer's protocol.

DNA Fragmentation Analysis. After treatment, cells were harvested for DNA as described previously (38). Briefly, $1–2 \times 10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris-HCl, 10 mM EDTA, and 0.5% SDS (w/v; pH 7.4)] to which RNase A (100 μg/ml) was added. After incubation for 2 h at 37°C, proteinase K (0.5 mg/ml) was added, and the lysates were heated to 56°C for 1 h. NaCl was then added (final concentration, 1 M), and lysates were incubated overnight at 4°C. Lysates were centrifuged at 15,000 $\times$ *g* for 30 min, and nucleic acids in the supernatant were precipitated in 2 volumes of ethanol with 50 mM sodium acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by ethidium bromide staining.

Western Blot Analysis. MCF-7 cells were grown for 2 days as described above, and then 5×10^6 cells were harvested in sonicating buffer [62.5 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, and 25 mg/ml aprotinin] and sonicated for 30 s. After centrifugation at 1,000 $\times$ *g* for 20 min, 50 μg of protein were resuspended in sample loading buffer [62.5 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromphenol blue], boiled for 3 min, and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with a 0.05% PBS-Tween-5% low-fat dry milk solution at 4°C overnight. The membrane was subsequently incubated with rabbit antisera (anti-Bcl-2, 1:4,000 dilution; anti-Bax, 1:4,000 dilution; anti-Bcl-X, 1:1,500 dilution; anti-Mcl-1, 1:2,000 dilution; and anti-Bak, 1:1,000 dilution) or with mouse anti-PARP-specific monoclonal antibody (1:5,000 dilution; PharMingen, San Diego, CA) and incubated for 2 h at room temperature. Blots were washed in PBS-Tween solution and incubated with goat antirabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat antimouse antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Oxford) for 30 min at room temperature. After four washes with PBS-Tween solution, immunoreactive proteins were detected using the

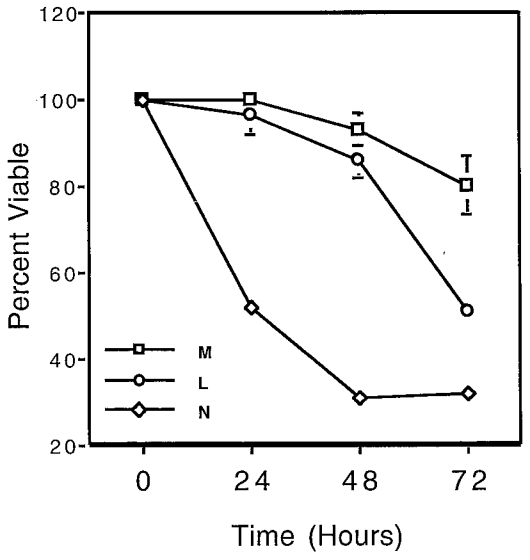


Fig. 2. Effects of TNF-α on MCF-7 cell viability. The percentage of cell death as measured by trypan blue staining at 24, 48, and 72 h of TNF-α treatment in MCF-7 cell variants M, L, and N is shown. *Error bars,* SD for three experiments. An absence of error bars represents <3% error between replicates.

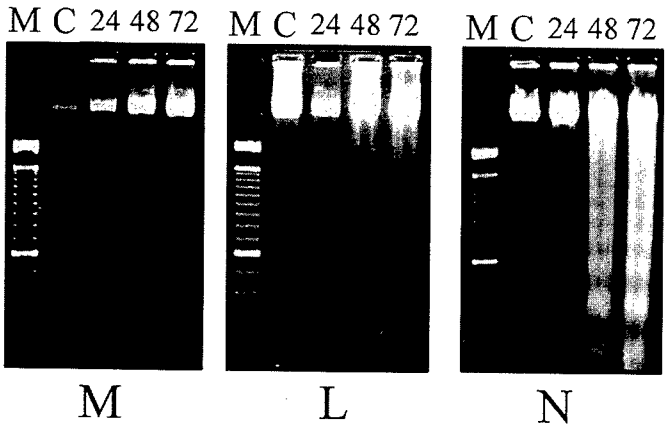


Fig. 3. TNF-α-induced DNA fragmentation of MCF-7 cells. An analysis of DNA fragmentation of MCF-7 cell variants M, L, and N by agarose gel (1.5%) electrophoresis at 24, 48, and 72 h after treatment with TNF-α (10 ng/ml) is shown.

enhanced chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad).

Flow Cytometry. Flow cytometric analysis of TNFR1 and TNFR2 was performed as described by Cai *et al.* (17). Briefly, 1×10^6 cells were harvested in PBS-EDTA and washed in 50% normal goat serum at 4°C for 15 min. Cells were washed in PBS-FBS (PBS with 1% FBS added) and incubated with 10 μg/ml mouse anti-TNF-α receptor antibodies (anti-TNFRp55 htr-9 and anti-TNFRp75 utr-1; BACHEM, Inc., King of Prussia, PA) in PBS-FBS at 4°C for 60 min. After this, the cells were washed three times in PBS-FBS and incubated with PE-conjugated goat antimouse IgG (1:40 dilution) in PBS-FBS at 4°C for 2 h. Cells were washed three times in PBS-FBS and analyzed using a Becton Dickinson FACStar flow cytometer. Excitation was at 488 nm (100 mW) using a coherent 6W argon-ion laser. For each cell, emission was measured using a photomultiplier with a 585 ± 42-nm band pass filter for phycoerythrin. Data were collected as 2,000 event list mode files and analyzed using LYSIS II (Becton Dickinson) software. Data represent duplicate counts of 2×10^3 cells, and statistical comparisons were made by Kolmogorov-Smirnov summation curves (39). Background fluorescence was determined using either unstained cells or cells stained using nonspecific mouse anti-α p65 monoclonal antibody (a kind gift of Kathleen Buckley, Department of Neuroscience, Harvard Medical School, Boston, MA).

Fluorescence Microscopy. For fluorescence microscopy, MCF-7 cells were seeded at 1×10^5 cells/ml in 10-cm^2 wells and treated with TNF-α (10 ng/ml) for 48 h. Samples were harvested, pelleted, and fixed in a solution of 10% formalin for 10 min and then washed with PBS and resuspended in a solution of propidium iodide in PBS (50 μg/ml). Cells were transferred to slides and visualized using a Zeiss Axioscope fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with appropriate filters.

Analysis of Ceramide. Ceramide was quantified by the DAG kinase assay as ^{32}P incorporated on the phosphorylation of ceramide to ceramide-1-phosphate by DAG kinase as described previously (40). Briefly, MCF-7 cells were treated with or without TNF-α (10 ng/ml) for the times indicated, washed in PBS, and fixed in ice-cold methanol. After extraction of the lipid, ceramide contained within the organic phase extract was resuspended in 20 μl of 7.5% α-octyl-β-glucopyranoside, 5 mM cardiolipin, and 1 mM diethylenetriamine pentaacetic acid (Sigma Chemical Co.). Thereafter, 40 μl of purified DAG kinase in enzyme buffer [20 mM Tris-HCl, 10 mM DTT, 1.5 M NaCl, 250 mM sucrose, and 15% glycerol (pH 7.4)] were added to the organic phase extract. Ten mM [γ-^{32}P]ATP (20 μl; 1000 dpm/pmol) in a buffer was added to start the reaction. After 30 min at 22°C, the reaction was stopped by the extraction of lipids with 1 ml of chloroform:methanol:hydrochloric acid (100:100:1, v/v). Buffered saline solution [170 μl; 135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES (pH 7.2)] and 30 μl of 100 mM EDTA were added. The lower organic phase was dried under N_2. Ceramide-1-phosphate was resolved by TLC using $CHCl_3$:CH_3OH:acetic acid (65:15:5, v/v) as a solvent and detected by autoradiography, and the incorporated ^{32}P was quantified by a phosphorimager (Fugi BAS1000; Fugi Medical Systems). The level of ceramide was determined by comparison to a concomitantly run standard curve composed of known amounts of ceramide.

RESULTS

Using three MCF-7 cell variants (M, L, and N) from established laboratories, we compared the effect of TNF-α on proliferation and viability. Under control conditions, different basal proliferation rates were observed among cell variants with doubling times of 30.8, 45, and 28.6 h for the M, L, and N cells, respectively (Fig. 1). The addition of TNF-α (10 ng/ml) to the medium inhibited basal proliferation in all three variants in a time-dependent manner. The most striking effect was observed in MCF-7 N cells, in which the number of viable cells per milliliter decreased by 50% from that of the control by 24 h. In the TNF-α-treated samples, trypan blue staining indicated that MCF-7 M cells retained >90% viability compared to that of the control on days 1 and 2 and 80% viability on day 3, whereas the viability of the TNF-α-treated MCF-7 N cells was 52, 31, and 32% of the control on days 1, 2, and 3. MCF-7 L cells treated with TNF-α were 97, 86, and 51% viable on days 1, 2, and 3 (Fig. 2). Additionally, the MTS viability assay revealed a dose-dependent effect of TNF-α (0.1–10 ng/ml) on MCF-7 cell variant viability and proliferation (data not shown). Consistent with the literature, 10 ng/ml was the optimal dose for both the induction of cell death (MCF-7 N cells) and the inhibition of proliferation (MCF-7 M and L cells; Ref. 17). These results suggest that MCF-7 N cells are highly sensitive to TNF-α-induced cytotoxic effects. Whereas the MCF-7 M cells were resistant to the cytotoxic effect of TNF-α, they still retained their sensitivity to the antiproliferative effect of TNF-α. TNF-α treatment of MCF-7 L cells resulted in an inhibition of proliferation and a delayed cytotoxic effect.

To determine whether the rapid loss of viability in the MCF-7 N variant on TNF-α treatment was due to apoptosis, DNA fragmentation analysis was performed. As expected from their retention of viability, the MCF-7 M variant did not undergo apoptosis in response to TNF-α

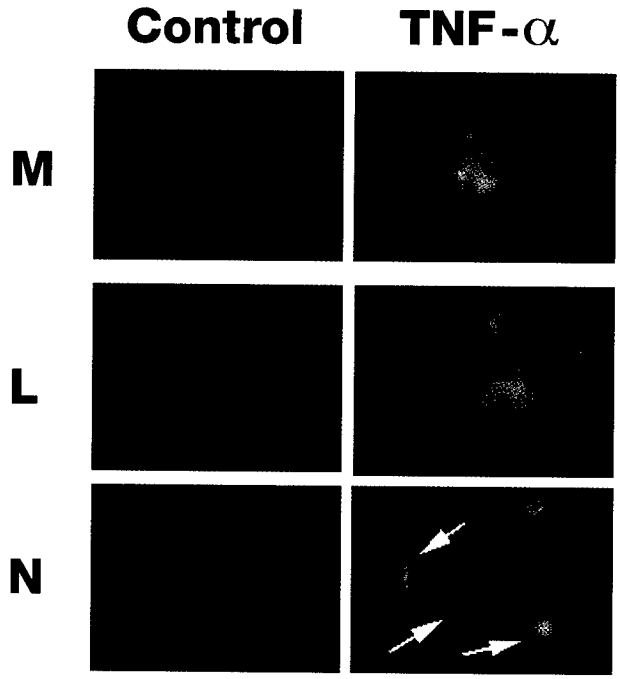


Fig. 4. TNF-α induced apoptosis of MCF-7 cells. Nuclear staining with 50 μg/ml propidium iodide revealed distinct nuclear condensation of the MCF-7 N variant (*arrows*) but not the M and L variants after 48 h of treatment with 10 ng/ml TNF-α.

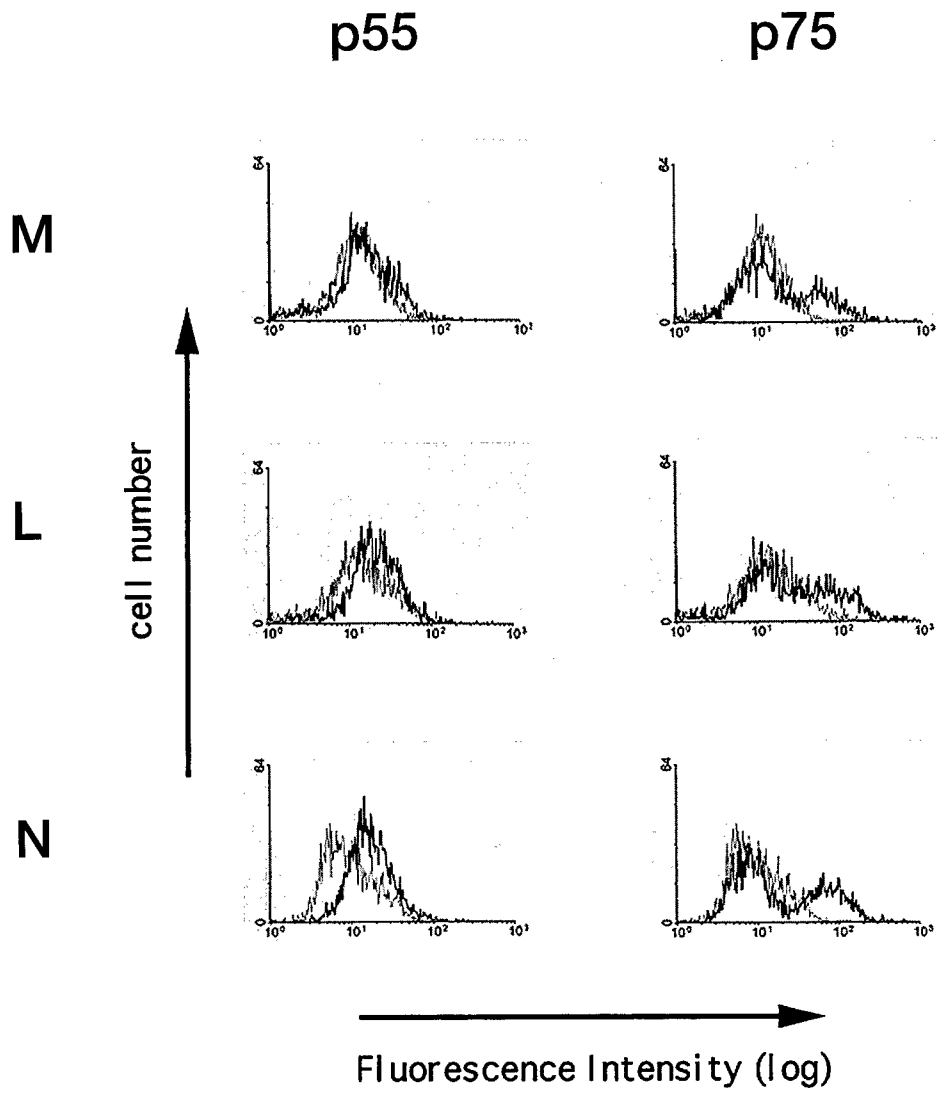


Fig. 5. Analysis of TNFR expression in MCF-7 cells. MCF-7 M, L, and N cells (1×10^6) were detached in PBS-EDTA, washed three times in PBS, and stained with antibodies specific to TNFR1 (p55) and TNFR2 (p75), respectively (*dark lines*), or with nonspecific antibodies to α-p65 (*gray lines*). Each panel is a frequency histogram depicting measurements on 2000 individual cells.

treatment (Fig. 3). However, TNF-α treatment resulted in DNA fragmentation as early as 24 h in the MCF-7 N variant and in moderate DNA fragmentation in the MCF-7 L stock by 72 h. These differences were confirmed by fluorescence microscopy (Fig. 4). Condensed nuclei were observed in TNF-α-treated MCF-7 N cells at 48 h and, as expected, were absent in the MCF-7 M and L variants.

TNFR expression was analyzed by flow cytometry with antibodies specific for TNFR1 (p55) or TNFR2 (p75). In Fig. 5, each curve is a frequency histogram of measurements on 2000 individual cells, with the number of cells in each of 1024 fluorescence channels displayed on a log scale. In each panel, the histogram obtained with the specific p55 or p75 antibody (*dark lines*) is overlaid with the curve derived from a nonspecific antibody to an irrelevant protein (*gray lines*). TNFR1 expression was determined to be 89 and 67% lower in the MCF-7 L and M cells as compared with that in the N cells. This finding suggests that the resistance of MCF-7 M and L cells may be due to their decreased expression of TNFR1. All three cell variants expressed similar levels of TNFR2 (*p75*).

Ceramide generation represents an early downstream event of TNF-α-induced signaling in numerous cell lines including MCF-7 cells (17, 29, 30). Ceramide also represents a key intermediate in the transduction of apoptotic signals from TNF-α as well as Fas, chemotherapeutic drugs, and γ-radiation (29, 30). The ability of water-soluble analogues of ceramide to induce apoptosis in MCF-7 cells further implicates ceramide as an important component in apoptotic signaling. TNF-α-induced ceramide generation was analyzed in the three MCF-7 cell variants to determine whether differences in SMase activity can account for the differential sensitivity to TNF-α-induced apoptosis. A rapid and transient increase in ceramide production was observed in the MCF-7 N variant, reaching a maximal level of 5.5 ± 0.56-fold over that of the control at 15 min with TNF-α (Fig. 6), whereas a 1.73 ± 0.37- and 1.42 ± 0.22-fold maximal increase in ceramide levels was observed in the M and L variants, respectively, at 15 min. All three cell variants possessed similar basal amounts of ceramide. Despite minimal expression of TNFR1, the MCF-7 M and L cells still responded, albeit weakly, to the ability of TNF-α to generate ceramide. Although substantial differences in TNFR1 expression exist between the MCF-7 M, L, and N cells, all cell variants express some degree of TNFR1, which suggests that other downstream events may also account for altered sensitivity to apoptosis.

The caspase family of proteases represents critical signaling intermediates and effectors of the apoptotic program (23, 32, 33). PARP is a proteolytic substrate for Asp-Glu-Val-Asp (DEVD)-specific caspases including CPP32/caspase 3 and caspase 7. Cleavage of PARP from its M_r 116,000 precursor to its M_r 29,000 and M_r 85,000 subunits is indicative of apoptosis and is a useful tool for the measurement of the time course of caspase activity (32, 33). Western blot analysis revealed caspase activity as early as 3 h in the TNF-α-

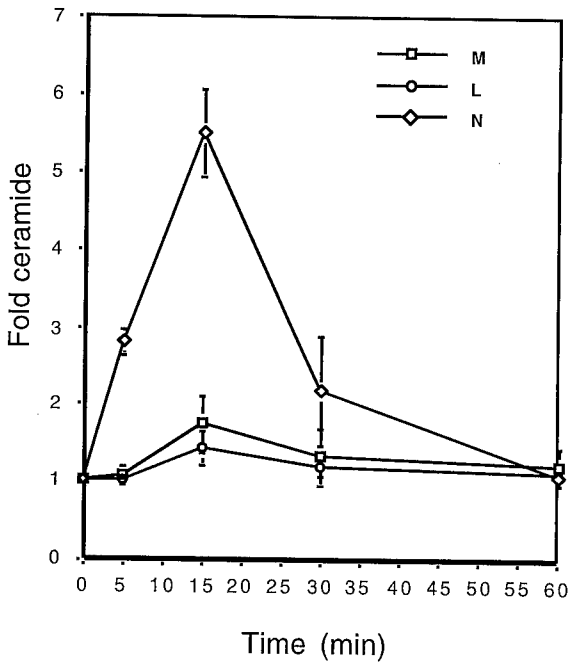


Fig. 6. Ceramide generation in MCF-7 cell variants with TNF-α treatment. MCF-7 M, L, and N cells (4×10^6) were treated with TNF-α (10 ng/ml) for the times shown above. Cells were harvested in ice-cold methanol; the lipid extraction and ceramide assay were performed as described in "Materials and Methods." The ceramide generated represents the fold change over control in nanograms of ceramide/milligrams of protein; *error bars*, SD of three independent experiments performed in duplicate.

sensitive N cells (Fig. 7). PARP cleavage in the moderately TNF-α-sensitive MCF-7 L cells was observed only at 6 h and was not observed in MCF-7 M cells.

bcl-2 proto-oncogene expression imparts considerable resistance to apoptosis induced by a variety of stimuli (34, 35). The relative expression of various members of the Bcl-2 family of proteins was analyzed in the three MCF-7 stocks by Western blot analysis (Fig. 8). Bcl-X, Mcl-1, and Bak protein expression was not appreciably different in the three stocks. However, striking differences were observed in the expression of Bcl-2 and Bax. Bcl-2 expression was 3.8 and 3.5 times higher in the apoptosis-resistant cell variants MCF-7 M and L, respectively, as compared to that in MCF-7 N. Bax expression was found to be 1.7- and 1.5-fold higher in the apoptosis-sensitive MCF-7 N variant as compared to that in the MCF-7 M and L variants.

DISCUSSION

Reported discrepancies exist concerning the apoptotic responses of MCF-7 cells to TNF-α and anti-Fas antibody treatment. Several studies have indicated that MCF-7 cells readily undergo apoptosis in response to TNF-α and anti-Fas (7, 8, 17). However, some reports have indicated that TNF-α and Fas only weakly induce apoptosis in MCF-7 cells (18–21). Others have shown that the cytotoxic *versus* the cytostatic effects of TNF-α depend on the media and serum conditions used to culture the MCF-7 cells (41). We report that under identical culture conditions, variations in apoptotic responses exist among three different MCF-7 cell strains obtained from established laboratories (M, L, and N). It was determined that proliferation of all three variants was inhibited by TNF-α, with the cell number of the MCF-7 N variant decreasing below control in parallel with a decrease in viability. The loss of viability in TNF-α-treated MCF-7 N cells was due to an induction of apoptosis observed as early as 24 h, whereas the MCF-7 L cells seemed moderately sensitive to the apoptotic effects of TNF-α only at 72 h. MCF-7 M cells were sensitive to the antiproliferative effect of TNF-α but resistant to the cytotoxic effects of TNF-α. Examination of the TNFR expression revealed a similar expression of p75 TNFR2 among all three cell variants. p55 TNFR1 was expressed at the highest levels in MCF-7 N cells and MCF-7 L cells, whereas MCF-7 M cells expressed the lowest levels of TNFR1. The decreased expression of TNFR1 in MCF-7 M cells may account in part for their lowered sensitivity to TNF-α apoptosis as well as a lowered genera-

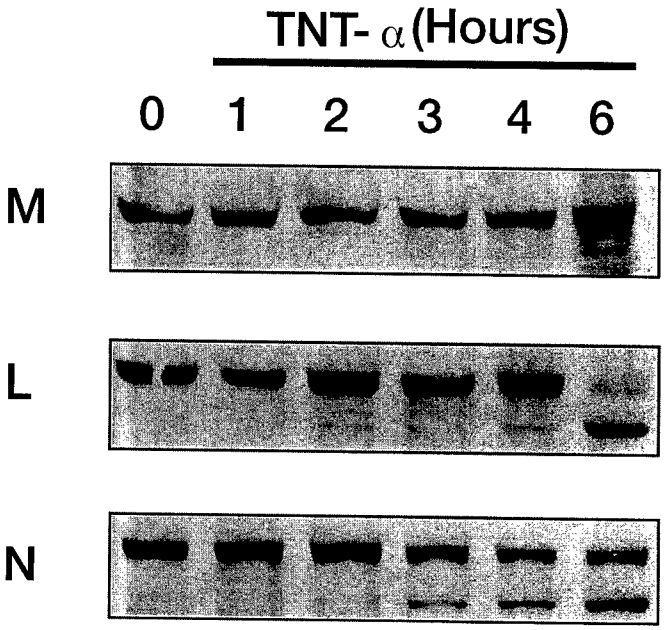


Fig. 7. Western blot analysis of PARP cleavage in MCF-7 cell variants. MCF-7 M, L, and N cells (2×10^6) were treated with TNF-α (10 ng/ml) for 1, 2, 3, 4, and 6 h. Cells were then harvested in PBS-EDTA and assayed for PARP cleavage as described in "Materials and Methods."

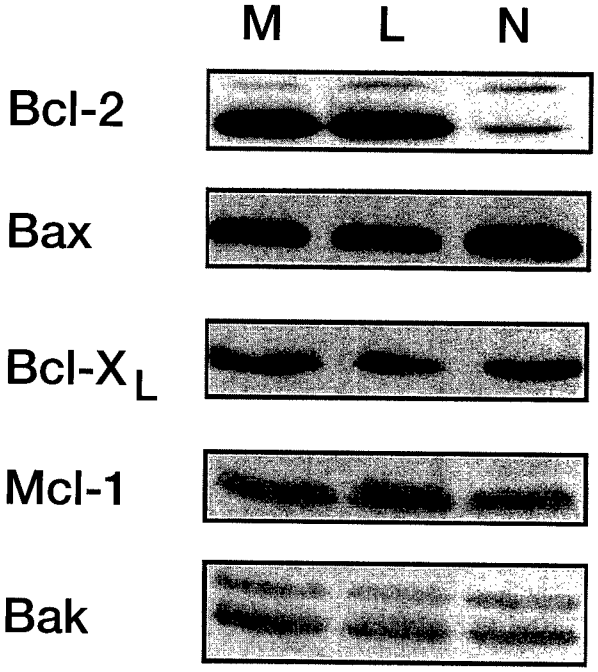


Fig. 8. Expression of Bcl-2 family proteins in MCF-7 cell variants. MCF-7 cell variants M, L, and N were grown for 2 days in normal media and harvested for Western blot analysis as described in "Materials and Methods" for the expression of Bcl-2, Bax, Bak, Bcl-X_L, and Mcl-1.

tion of ceramide. It is possible that despite lowered expression, TNFR1 or even TNFR2 may provide the antiproliferative signal in these cells. Given the role of ceramide in the inhibition of proliferation, the 1.7-fold increase in ceramide in the M cells may be sufficient for the suppression of cell proliferation but insufficient to induce apoptosis. We cannot rule out the possibility that altered expression or activation of TRADD, FADD, FLICE, or other proteins in the TNF signaling cascade may account for the inability to activate apoptosis in the M cells.

Examination of several members of the Bcl-2 family of apoptosis-regulating proteins suggests that the intrinsic resistance of the M cells and the delayed apoptotic DNA laddering and protease activation in the MCF-7 L cells as compared to the N variant were correlated with a higher expression of Bcl-2 and a lower expression of Bax. Many studies confirm that an increase in the expression of Bcl-2 correlates with resistance to apoptosis induced by a number of agents (34, 35). However, contradictory reports exist as to the ability of Bcl-2 or Bcl-X_L expression to inhibit TNF-α-induced apoptosis in MCF-7 cells. Vanhaesebroeck *et al.* (42) showed that overexpression of Bcl-2 in MCF-7 cells failed to offer a survival advantage to treatment with TNF-α. Conversely, Jaattela *et al.* (43) showed that overexpression of Bcl-2 and Bcl-X_L was correlated with an increased resistance to TNF-α-induced apoptosis. Again, these reported differences may be due to the individual MCF-7 cell variants used by each laboratory and may potentially be due to the variations in constitutive expression of other members of the Bcl-2 family, such as Bax. Overexpression of Bax or Bcl-X_S in MCF-7 cells resistant to chemotherapeutic drug treatment, serum starvation, and Fas-induced apoptosis has been shown to sensitize these cells to the induction of apoptosis (44–46). Thus, cells expressing high levels of Bax may not be as resistant to apoptosis, even when overexpressing Bcl-2. However, the Bcl-2 family of proteins may not account for all of the differences in apoptotic sensitivity reported here. Both the M and L stocks express similar levels of Bcl-2, Bax, Bak, Bcl-X, and Mcl-1; however, the L cells undergo apoptosis in response to TNF-α, whereas the M cells are resistant, suggesting that other differences within variants of MCF-7 cells will affect the antiapoptotic role of Bcl-2. Reports have indicated that Bcl-2 does not block ceramide generation but does inhibit ceramide analogue-induced apoptosis (47). Given the ability of Bcl-2 to block protease activation through the inhibition of cytochrome *c* release from the mitochondria (36, 37), the increased Bcl-2 expression and decreased Bax expression in MCF-7 L cells account for the delayed activation of PARP-specific caspases but not for the suppressed generation of ceramide.

Our results suggest a potential molecular basis for the differences in susceptibility to apoptosis among MCF-7 breast cancer cell variants. The increased generation of ceramide in the most apoptosis-sensitive variant cells (MCF-7 N) may account for their response to TNF-α as compared to the antiproliferative action of TNF-α in the less apoptosis-sensitive variants (MCF-7 M and L). This decreased ceramide generation may be due in part to decreased expression of TNFR1, as in the MCF-7 M cells, or possibly to an alteration in the ability of TNF-α to activate SMases, which may be the case in MCF-7 L cells. In MCF-7 cells, ceramide generation is early and transient, suggesting that its SMase activation is not a result of the apoptotic process but an early signaling intermediate. Gamen *et al.* (48) implicated CPP32/caspase 3 in the regulation of Fas-induced ceramide generation but not TNF-α-induced ceramide generation. Additionally, it was shown that REAPER-induced ceramide generation occurring at 1 h or later is blocked by an ICE-like protease inhibitor (49). We cannot rule out the possibility that ceramide generation may be mediated by events subsequent to FLICE/MACH1/caspase 8 or early caspase-dependent activation. In summary, our data indicate that the sensitivity of MCF-7 cells to apoptosis induced by TNF-α and other agents differs depending on the origin of the cells. Given the extensive use of MCF-7 cells as an ER-positive breast cancer model and a system for studying apoptotic signaling, the constitutive expression and regulation of apoptotic signaling molecules are therefore an important consideration.

ACKNOWLEDGMENTS

We thank Drs. William Toscano, Louise Nutter, and Stephen M. Hill for provision of the MCF-7 cell variants; Drs. Kevin Lorick, William Hartley, and William Toscano for access to and assistance with the fluorescence microscope; and William Toscano, Aline B. Scandurro, and Bridgette M. Collins-Burow for critical reading of the manuscript.

REFERENCES

1. Soule, H., Vazquez, J., Long, A., Albert, S., and Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Cancer Inst., *51:* 1409–1413, 1973.
2. Levenson, A. S., and Jordan, V. C. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res., *57:* 3071–3078, 1997.
3. Osbourne, C. K., Hobbs, K., and Trent, J. M. Biological differences among MCF-7 human breast cancer cell lines from different laboratories. Breast Cancer Res. Treat., *9:* 111–121, 1987.
4. Klotz, D. M., Castles, C. G., Fuqua, S. A. W., Spriggs, L. L., and Hill, S. M. Differential expression of wild-type and variant ER mRNAs by stocks of MCF-7 breast cancer cells may account for differences in estrogen responsiveness. Biochem. Biophys. Res. Commun., *210:* 609–615, 1995.
5. Villalobos, M., Olea, N., Brotons, J. A., Olea-Serrano, M. F., Ruiz de Almodovar, J. M., and Peraza, V. The E-screen assay: a comparison of different MCF-7 cell stocks. Environ. Health Perspect., *103:* 844–850, 1995.
6. Kiguchi, K., Glense, D., Chubb, C. H., Fujiki, H., and Huberman, E. Differential induction of apoptosis in human breast tumor cells by okadaic acid and related inhibitors of protein phosphatases 1 and 2A. Cell Growth Differ., *5:* 995–1004, 1994.
7. Tewari, M., and Dixit, V. M. Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus *crm*A gene product. J. Biol. Chem., *270:* 3255–3260, 1995.
8. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J. Biol. Chem., *271:* 4961–4965, 1996.
9. Liu, Y., Lee, M., Wang, H., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X. K. Retinoic acid receptor β mediates the growth inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. Mol. Cell. Biol., *16:* 1138–1149, 1996.
10. Elstner, E., Linker-Israeli, M., Said, J., Umeil, T., de Vos, S., Shintaku, I. P., Heber, D., Binderup, L., Uskokovic, M., and Koeffler, H. P. 20-epi-vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res., *55:* 2822–2830, 1995.
11. James, S. Y., Mackay, A. G., and Colston, K. W. Vitamin D derivatives in combination with 9-*cis*-retinoic acid promotes active cell death in breast cancer cells. J. Mol. Endocrinol., *14:* 391–394, 1995.
12. Perry, R. R., Kang, Y., and Greaves, B. Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. Ann. Surg. Oncol., *2:* 238–245, 1995.
13. Wang, T. T. Y., and Phang, J. M. Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. Cancer Res., *55:* 2487–2489, 1995.
14. Kyprianou, N., English, H. F., Davidson, N. E., and Isaacs, J. T. Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res., *51:* 162–166, 1991.
15. Blagosklonny, M. V., Schulte, T., Nguyen, P., Trepel, J., and Neckers, L. M. Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involves c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. Cancer Res., *56:* 1851–1854, 1996.
16. Jeoung, D., Tang, B., and Sonenberg, M. Effects of tumor necrosis factor-α on antimitogenicity and cell cycle-dependent proteins in MCF-7 cells. J. Biol. Chem., *270:* 18367–18373, 1995.
17. Cai, Z., Bettaieb, A., Mahdani, N. E., Legres, L. G., Stancou, R., Masliah, J., and Chouaib, S. Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF-7 cells to tumor necrosis factor-α-mediated cytotoxicity. J. Biol. Chem., *272:* 6918–6926, 1997.
18. Belizario, J. E., Tilly, J. L., and Sherwood, S. W. Caffeine potentiates the lethality of tumor necrosis factor in cancer cells. Br. J. Cancer, *67:* 1229–1235, 1993.
19. Malorni, W., Rainaldi, G., Tritarelli, E., Rivabene, R., Cianfriglia, M., Lehnert, M., Donelli, G., Peschele, C., and Testa, U. Tumor necrosis factor α is a powerful apoptotic inducer in lymphoid leukemia cells expressing p-170 glycoprotein. Int. J. Cancer, *67:* 238–247, 1996.
20. Kean, M. M., Ettenberg, S. A., Lowrey, G. A., Russell, E. K., and Lipkowitz, S. Fas expression and function in normal and malignant breast cell lines. Cancer Res., *56:* 4791–4798, 1996.

21. Tang, P., Hung, M., and Klostergard, J. TNF cytotoxicity: effects of HER-2/neu expression and inhibitors of ADP-ribosylation. Lymphokine Cytokine Res., *13:* 117–123, 1994.
22. Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. Two tumor necrosis factor receptors: structure and function. Trends Cell Biol., *5:* 392–399, 1995.
23. Nagata, S. Apoptosis by death factor. Cell, *88:* 355–365, 1997.
24. Hsu, H., Xiong, J., and Goeddel, D. V. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell, *81:* 495–504, 1995.
25. Hsu, H., Huang J., Shu, H-B., Baichwal, V., and Goeddel, D. V. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity, *4:* 387–396, 1996.
26. Hsu, H., Shu, H-B., Pan, M-G., and Goeddel, D. V. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell, *84:* 299–308, 1996.
27. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer P. H., Peter, M. E., and Dixit, V. M. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (FAS/APO-1) death-inducing signaling complex. Cell, *85:* 817–827, 1996.
28. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1-and TNF receptor-induced cell death. Cell, *85:* 803–815, 1996.
29. Hannun, Y. A. Functions of ceramide in coordinating cellular responses to stress. Science (Washington DC), *274:* 1855–1859, 1996.
30. Peña, L. A., Fuks, Z., and Kolesnick, R. Stress-induced apoptosis and the sphingomyelin pathway. Biochem. Pharmacol., *53:* 615–621, 1997.
31. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell, *78:* 1005–1015, 1994.
32. Nicholson, D. W., and Thornberry, N. A. Caspases: killer proteases. Trends Biochem. Sci., *22:* 299–306, 1997.
33. Cohen, G. M. Caspases: the executioners of apoptosis. Biochem. J., *326:* 1–16, 1997.
34. Reed, J. C. Regulation of apoptosis by Bcl-2 family of proteins and its role in cancer and chemoresistance. Curr. Opin. Oncol., *7:* 541–546, 1995.
35. Korsmeyer, S. J. Regulators of cell death. Trends Genet., *11:* 101–105, 1995.
36. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T., Jones, D. P., and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science (Washington DC), *275:* 1129–1132, 1997.
37. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science (Washington DC), *275:* 1132–1136, 1997.
38. Subramanian, T., Tarodi, B., and Chinnadurai, G. p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: suppression by E1B 19K and Bcl-2 proteins. Cell Growth Differ., *6:* 131–137, 1995.
39. Young, I. T. Proof without prejudice: use of the the Kolmogorov-Smirnov test for the analysis of histograms for flow systems and other sources. J. Histochem. Cytochem., *25:* 935–941, 1977.
40. Clejan, S., Dotson, R. S., Wolf, E. W., Corb, M. P., and Ide, C. F. Morphological differentiation of N1E-115 neuroblastoma cells by dimethyl sulfoxide activation of lipid second messengers. Exp. Cell Res., *224:* 16–27, 1996.
41. Pagliacci, M. C., Fumi, G., Migliorati, G., Gingnani, F., Riccardi, C., and Nicoletti, I. Cytostatic and cytotoxic effects of tumor necrosis factor α on MCF-7 human breast tumor cells are differentially inhibited by glucocorticoid hormones. Lymphokine Cytokine Res., *12:* 439–447, 1993.
42. Vanhaesebroeck, B., Reed, J. C., De Valck, D., Grooten, J., Miyashita, T., Tanaka, S., Beyaert, R., Frans, V. R., and Fiers, W. Effect of *bcl*-2 proto-oncogene expression on cellular sensitivity to tumor necrosis factor-mediated cytotoxicity. Oncogene, *8:* 1075–1081, 1993.
43. Jaattela, M., Benedict, M., Tewari, M., Shayman, J. A., and Dixit, V. M. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A_2 in breast carcinoma cells. Oncogene, *10:* 2297–2305, 1995.
44. Sumantran, V. N., Ealovega, M. W., Nunez, G., Clarke, M. F., and Wicha, M. S. Overexpression of Bcl-X_S sensitizes MCF-7 cells to chemotherapy-induced apoptosis. Cancer Res., *55:* 2507–2510, 1995.
45. Wagener, C., Bargou, R. C., Daniel, P. T., Bommert, K., Mapara, M. Y., Royer, H. D., and Dorken, B. Induction of the death-promoting gene Bax-α sensitizes cultured breast cancer cells to drug induced apoptosis. Int. J. Cancer, *67:* 138–141, 1996.
46. Bargou, R. C., Wagner, C., Bommert, K., Mapara, M. Y., Daniel, P. T., Arnold, W., Dietel, M., Guski, H., Feller, A., Royer, H. D., and Dorken, B. Overexpression of the death-promoting gene bax-α which is down-regulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. J. Clin. Investig., *97:* 2651–2659, 1996.
47. Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M., and Hannun, Y. A. Bcl-2 interrupts the ceramide-mediated pathway of cell death. Proc. Natl. Acad. Sci. USA, *93:* 5325–5328, 1996.
48. Gamen, S., Marzo, I., Anel, A., Piñeiro, A., and Naval, J. CPP32 inhibition prevents Fas-induced ceramide generation and apoptosis in human cells. FEBS Lett., *390:* 233–237, 1996.
49. Pronks, P. J., Ramer, K., Amiri, P., and Williams, L. T. Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. Science (Washington DC), *271:* 808–810, 1996.